AFLATOXIN ELIMINATION WORKSHOP

Fresno, California

October 28 - 29, 1996

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Throughout the world, aflatoxin is considered one of the most serious food safety problems. Chronic problems with aflatoxin contamination occur in the southern U.S. in cottonseed, corn, peanuts and tree nuts. However, the impact of aflatoxin contamination on the agricultural economy is especially devastating during drought years when aflatoxin affects the Mid West corn belt. Estimated economic losses in years of major aflatoxin outbreaks have been in the hundreds of millions of dollars.

The Aflatoxin Elimination Workshops have served to bring together the Agricultural Research Service (ARS, USDA) and university scientists and representatives of the peanut, cottonseed, corn and tree nut industries in a unique cooperative effort with the mission to develop aflatoxin control strategies through research and development. The ultimate goal of this effort is to facilitate the commercial implementation of technologies to eliminate the aflatoxin contamination problem in the U. S. marketplace by the turn of the millennium. Most of the research to eliminate aflatoxin is conducted by ARS, however, an important addition to this effort is a competitive award program provided by Congressional appropriations to bring to the research effort the talents of university scientists in cooperation with ARS.

Scientists realized over a decade ago that the aflatoxin problem cannot be solved solely by conventional technologies utilized routinely to control the more "typical" plant pathogens. Aflatoxin contamination is a result of fungal infection of host plants by a unique class of microorganisms adapted to subsist saprophytically on organic debris in the field or to infect and produce aflatoxin in living plant tissues. Classical plant disease prevention methods developed to control very fastidious plant pathogens have been generally unsuccessful in excluding aflatoxin producing fungi from their relatively broad ecological niche. The realization of the unique nature of the aflatoxin problem and that novel technologies that will be required for its control became a focal point of discussion during strategy development sessions of the first Aflatoxin Elimination Workshop in 1988. Two areas of research and development based on the biology and ecology of *Aspergillus flavus*-group fungi were suggested: 1) novel genetic engineering and/or marker-based breeding methods to enhance general antifungal resistance in crops, and 2) the isolation and formulation of special fungi for use in biocontrol. These biocontrol fungi belong to the *Aspergillus flavus*-class of fungi but do not produce aflatoxin and have the capability to occupy the same ecological niche in the field and outcompete harmful toxin-producing fungi.

The vision of participants in the first Aflatoxin Elimination Workshop has been confirmed by the rapid progress reported in subsequent workshops in developing practical, commercially viable aflatoxin control procedures, based almost entirely on the concepts generally established during the first workshop. For example, aflatoxin levels were significantly reduced in field plots of Arizona-grown cottonseed and Georgia-grown peanut through application of new biocontrol formulations consisting of atoxigenic strains of *Aspergillus flavus*-group fungi. The IR-4 Biopesticide Program recently assisted in obtaining the EPA Experimental Use Permit under which large scale field testing of one of these biocontrol formulation was begun in Arizona cotton fields during the Summer of 1996.

Although biologically-based technologies are important themes of the workshop, improvement of crop management and handling practices has also been emphasized. Improvements were reported

at the 1996 workshop in handling practices such as utilizing new sorting methods to exclude insect and/or fungally damaged nuts. These methods to exclude damaged nuts, which are based on X-ray and color scanning technologies, may be the option of choice to control aflatoxin contamination in certain high value crops, such as pistachios and other tree nuts. Sessions also strongly reinforced the need to use such crop management and handling practices as optimum time of harvest, irrigation, insect control, and mechanical exclusion of bad seed/kernels to eliminate aflatoxin in the final food or feed product. These careful crop management/handling practices may never be replaced even with the use of powerful new biologically-based technologies.

Economically feasible and efficient methods to eliminate the aflatoxin problem in many crops will likely be linked to the introduction of resistant crop germplasm to the commercial market. Corn and peanut, especially, have a large amount of natural genetic diversity with respect to fungal infection response and levels of aflatoxin obtained. Naturally resistant corn and peanut germplasm not only provides a source of resistance, but also nature's examples of specific chemical and structural barrier requirements for resistance to fungal invasion and aflatoxin contamination. The identification of actual mechanisms and traits for plant resistance against fungal infection and aflatoxin synthetic processes was reported at the workshop; thus, confidence is building that marker-based plant breeding can be used to successfully introduce resistance to A. flavus into commercial crop lines. In addition, we have identified potent antifungal genes to use in a technology that is now becoming routine--the genetic engineering of cotton, peanut and walnut to prevent invasion of these crops by aflatoxin producing fungi.

In an extremely important breakthrough in basic research, scientists attending the workshop reported the characterization of the actual genetic "locus" in *Aspergillus* which governs aflatoxin production. This research has caught the eye of the fungal genetics community around the world. Research has already begun to breed or engineer plants resistant to aflatoxin contamination through screening for and enhancing production of phytochemicals that interfere with expression of the aflatoxin locus. DNA associated with this locus critical for aflatoxin formation has been deleted to produce a new generation of atoxigenic fungi for possible use in biocontrol formulations in the future. These investigations illustrate how basic research, targeted toward practical application, is critical in finding solutions to agricultural problems.

Research information summarized in this 1996 workshop proceedings provides the technological foundation for the multiple strategies currently being investigated to eliminate aflatoxin, thus leading to the well grounded optimism that solutions to this serious food safety problem will be available by the beginning of the next century. This innovative aflatoxin control technology under development was made possible only by the ingenious application of research information on the nature of *Aspergillus flavus*-group fungi and of the aflatoxin contamination process, knowledge which has been provided over the years by scientists attending the workshop.

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AFLATOXIN ELIMINATION WORKSHOPS

New Orleans, LA	1988
Peoria, IL	1989
St. Louis, MO	1990
Atlanta, GA	1991
Fresno, CA	1992
Little Rock, AR	1993
Atlanta, GA	1995
Fresno, CA	1996
Memphis, TN	1997

COOPERATING COMMODITY GROUPS

PEANUTS:	National Peanut Council
CORN:	National Corn Growers Association American Corn Millers Federation Corn Refiners Association
COTTONSEED:	National Cottonseed Products Association National Cotton Council
TREE NUTS:	Almond Board of California California Pistachio Commission DFA of California

TABLE OF CONTENTS

AFLATOXIN ELIMINATION WORKSHOP
ABSTRACTS OF PRESENTATIONS

POSTER PRESENTATIONS:

CROP RESISTANCE - GENETIC ENGINEERING

PLATFORM PRESENTATIONS (Special Platform Poster Format):

For Panel Discussions, Panelists Combined the following Two Workshop Sessions:

1. **Molecular Mechanisms Governing Aflatoxin Pathway Gene Expression**
2. **Potential Use of Native Plant Compounds for the Prevention of Aflatoxin Contamination**

MOLECULAR MECHANISMS GOVERNING AFLATOXIN PATHWAY GENE EXPRESSION

PLATFORM PRESENTATIONS:

POSTER PRESENTATION:

MICROBIAL ECOLOGY

PLATFORM PRESENTATIONS

PANEL DISCUSSION

PANEL DISCUSSION TITLE: An Integrated Approach to Aflatoxin Control Based on the Epidemiology of Naturally Occurring Toxigenic and Atoxigenic Strains of *Aspergillus flavus/parasiticus* Infecting Crops

PANEL MEMBERS: Merritt Nelson (Chair), J. Caceres, Dick Cole, Peter Cotty and Joe Dorner

SUMMARY OF PANEL DISCUSSION: The initial topic posed by Merritt Nelson to Joe Dorner and Peter Cotty referred to the quantity of biological control material that is needed for field applications. Peter Cotty's work has used selected atoxigenic strains of *A. flavus*, while Joe Dorner's work is more focused on using induced mutants of *A. flavus* and related species as biological competitors.

Dorner said that in peanut experimental plots various rates had been used. Best results were obtained with the 500 lb. per acre rate. Dorner and associates predict that as the product development proceeds they will possibly get down to a start rate of 50 to 100 pounds. Cotty is currently using 10 lbs/acre in field tests in cotton, but previously in field trials 5 lbs/acre gave good control. It is unknown at this point why different rates of biocontrol product appear to be necessary on the two different crops (cotton and peanut).

The biological control agents used by Dorner and associates in peanuts include a UV induced mutant of a wild type *A. flavus*. This is a colored mutant convenient to monitor populations. They also developed a very aggressive wild type *A. parasiticus* UV induced mutant which was also aflatoxin non-producing. Neither of these mutants produce cyclopiazonic acid. All strains produce kojic acid.

How long do fungal biocontrol populations last? Research results in both laboratories (Dorner and Cotty) suggest that biocontrol populations decline very rapidly initially. It is presumed, that it may be necessary to regularly renew the biological control agents by repeated applications. Attempts to formulate products with shelf life, price, applicability and effectiveness is a major goal of both projects. The issue was raised, why not use cornflower as a carrier, since particle size can be adjusted, and it may be quite economical. Rice grains may be a practical means of formulating biopesticides for application. Size can be adjusted so that small particles can provide better coverage but active material then is not as well protected and shelf life may be shortened. Cotty indicated that coverage did not seem to be a big concern in cotton because conidia produced on inoculum seem to rapidly spread and compensate for gaps.

The quantity, location and timing of applications of biopesticides was discussed. It may be necessary to initially apply large quantities to a field initially with a history of heavy aflatoxin contamination. This may be followed with lighter applications in succeeding years to maintain effective populations. One key to successful applications will be finding a niche in which the particular agent may prosper and compete effectively with the wild type. Based on analysis of crop sequencing patterns on populations of *A. flavus* in cotton culture in Eastern Yuma County, Arizona, cropping patterns affect not only the total population magnitude but also differentially the level of the L and S strain populations.

We need to modify the expectation that the results must involve a dramatic reduction in the populations of the wild type *A. flavus* when in fact only a modest decrease in such populations may be effective in reducing aflatoxin contamination in the crop. Thus, a smaller quantity of the inoculum may be sufficient. Even though the dose response curve of aflatoxin versus biocontrol agent is extremely difficult to measure, it was concluded that moderate doses should be considered in the determination of application strategies.

Possible destruction of the formulation following application may be a problem. Animals, including insects, small mammals, birds as well as fungi and other microorganisms, may find these materials to be a good food base. Since these materials are applied at a time optimal for sporulation, the main dispersal form (conidia) of the agents themselves might easily be prey to animals and microbes. In the worst cases the applied biocontrol formulation may be destroyed in five to seven days.

Another observation was that large scale applications may not require as large a quantity per unit area as indicated by small plot work. Of necessity small plots are close together and there may be an interaction between treatments with more pressure put on treated replications by nearby untreated plots.

In certain years in western Arizona, isolates belonging to the S strain of *A. flavus* may cause most of the contamination of cottonseed. However, the typical atoxigenic L strain isolates are apparently better adapted than the S strain to infection of the crop and dispersal within the canopy. Differing adaptations of biocontrol fungal strains may explain differences in levels of inoculum required to be effective depending upon the nature of the particular fungal strain and/or crop system (e. g. peanut vs. cotton). In summary, an integrated approach to aflatoxin control based on the epidemiology of naturally occurring toxigenic and atoxigenic strains of *A. flavus/parasiticus* infecting crops may contain some or all of the following elements:

1. Selection of isolates that can be developed into competitive agents for application to crops.

2. Development of proper formulations and application strategies for the utilization of biopesticides used for determining where application may be most effective.

3. Understanding of the spatial distribution of wild type strains on both a field and regional basis. This is particularly important as baseline information to evaluate the effectiveness of biopesticides used.

4. Understanding of the influence of cropping patterns and other biological and physical environmental parameters on the population magnitude of *A. flavus/parasiticus* strains required for effective biological control.

5. Development of the baseline dosage response information in order to assure adequate evaluations of the economics and commercial applicability of biocontrol technologies. Overall, the variation in the spatial characteristics and magnitude of the aflatoxin contamination problem and the potential variation in the results of application of biological control agents suggests that accurate evaluations be made over a period of time before making conclusions regarding effectiveness.

THE EPA APPROVED EXPERIMENTAL USE PROGRAM FOR *ASPERGILLUS FLAVUS* AF 36

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An Experimental Use Permit (EUP) for use of *Aspergillus flavus* AF36 in the management of aflatoxin contamination of cottonseed in Arizona was granted by the U.S. Environmental Protection Agency on May 20, 1996. Also granted was a Temporary Exemption from Tolerance for *Aspergillus flavus* AF36 on commercial cottonseed. The EUP expires in May 1999. Prior to these actions, the EPA required an acute oral toxicity test on *Aspergillus flavus* AF36. The test was performed on rats by a contract laboratory and no toxicity was detected.

The experimental program outlined in the Experimental Use Permit calls for 120 acres to be treated in 1996 and 500 to be treated in both 1997 and 1998. In collaboration with gins and growers in Yuma County, Arizona, an experimental plan was designed to comply with this program. The design calls for the treating of fields in defined regions of the Yuma and Gila Valleys. Planned studies will also develop methods for evaluating efficacy that are acceptable to the cotton community. Long term and area wide influences of applications will also be assessed.

Mass production of inoculum production was achieved in the laboratory and the manufacturing process and quality control program were approved by the EPA. The manufacture entailed mixing a conidial suspension with sterile wheat on a roller until free liquid was absorbed (3 h) followed by incubation for 20 to 22 h at 31°C and drying at 56°C. Twelve hundred pounds of inoculum were manufactured in 1996. Wheat prepared in this manner retained viability at room temperature for over a year and was stable for 1 month when held at temperatures up to 50°C. The product was shipped to growers in heat sealed bags under EPA label and was stored with other agrochemicals until use.

The first treatments to commercial fields were initiated in June 1996. The product was applied by growers at lay-by with modified Gandy boxes to three commercial fields. The extent of Gandy box modification varied with grower. The primary modification was to the drop tube in order to facilitate delivery under the canopy. Commercial application succeeded in delivering on average 56% of the colonized wheat to the top of the beds under the canopy. Thirty-four percent was delivered to the bed face and 11% fell in the furrow. Significant quantities of the product were not washed down the furrows at irrigation. Six days after irrigation 92% of the applied wheat had yielded *A. flavus* conidia. Wheat sporulated similarly at the bottom of furrows and on the top of beds. Samples of the sporulating wheat seed were collected from the field; vegetative compatibility tests were performed on 30 isolates from these samples. All isolates were confirmed to be the intended applied strain. Prior to treatment, soils in treated, control, and monitoring fields were sampled. A total of 1,560 *Aspergillus flavus* isolates were recovered from the samples. Genetic analyses of these isolates are being performed in order to determine the distribution of *Aspergillus flavus* AF36 prior to treatment.

LANDSCAPE ANALYSIS OF *ASPERGILLUS FLAVUS* SOIL SURFACE POPULATIONS

Merritt R. Nelson[1], D. M. Bigelow[1], T. V. Orum[1], D. R. Howell[2] and P.J. Cotty[3]
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The S and L strains of *Aspergillus flavus* differ greatly in aflatoxin production in Arizona. In previous work, spatial and temporal patterns of strain S incidence and total *A. flavus* population magnitude were studied using a nested sampling design. Conclusions from that work include the following: 1) both *A. flavus* population magnitude and strain S incidence peak during the summer; 2) the Texas Hill sub-region had significantly greater strain S incidence than other areas; 3) the most important scale for further study is among fields within areas (1 to 5 km); and 4) there is a need to sample adjacent fields to see if patches of low strain S incidence extend beyond field boundaries.

Fields separated by only 1 to 5 km were shown to differ significantly in S strain incidence. For example, in the North Gila Valley, field 5 averaged 9% strain S incidence over four sampling dates from March 1995 through March 1996 compared with field 28 (about 2 km away) which averaged 51% strain S incidence over the same four sampling dates. These differences continued in July 1996 (field 5, 12% strain S; field 28, 66% strain S) and were observed to extend into adjacent fields. Two newly sampled fields adjacent to field 5 averaged 25% strain S, whereas two fields adjacent to field 28 averaged 52% strain S. Groups of adjoining fields within several areas (North Gila, Dome Valley, and Texas Hill) were shown to differ in S strain incidence in a statistically significant manner despite within group differences in crop sequences. Thus, temporal and spatial consistency in strain composition transcends field boundaries and crop sequences.

Sub-regional differences continued to be observed. Texas Hill (16 fields) averaged 75% strain S while other Yuma County areas (18 fields) averaged 42% strain S. In the Texas Hill sub-region, two relatively low spots were found. Both occurred within 1 km of the Gila River. A detailed analysis of crop sequences in the Texas Hill sub-region failed to shed light on strain composition patterns. *A. flavus* population magnitude, on the other hand, differed under contrasting crop sequences. In five Texas Hill fields where perennial bermuda grass has grown for four consecutive years, the *A. flavus* population magnitude (98 propagules/g) was significantly less than in five nearby cotton fields (860 propagules/g). Throughout Yuma County, the highest population magnitudes were seen in five fields that were fallow after wheat (5051 propagules/g), followed by fifteen cotton fields (2248 propagules/g), and five sudan grass fields (873 propagules/g).

Contrasting spatial models are being considered for population magnitude and strain composition. Patches of similar *A. flavus* population magnitude are aligned with fields of similar recent crop histories and may relate to available organic matter or to crop management (soil moisture levels, tillage, etc.) There are no obvious magnitude differences at the sub-regional scale (20-100 km) within Yuma County. Patches of similar strain composition extend beyond field boundaries and are more difficult to explain. It may be that pioneering colonies of particular strains tend to dominate in local areas for a while. Sub-regional differences suggest yet to be determined factors may give strain S colonies an advantage in the Texas Hill area.

DEVELOPMENT OF COMMERCIAL FORMULATIONS FOR DELIVERY OF AFLATOXIN BIOCONTROL AGENTS TO PEANUTS

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Studies in previous years have shown the effectiveness of applying non-toxigenic color mutants of *Aspergillus flavus* and *A. parasiticus* to peanut soil in reducing preharvest aflatoxin contamination of peanuts. A 99.9% reduction in aflatoxin contamination was achieved in plots that were treated for 2 years compared with non-treated control plots. Those studies utilized rice that was infested with the non-toxigenic color mutants for inoculating soil.

Whereas the rice formulation has proven to be an effective delivery system for biocompetitive fungal strains to soil, it does not lend itself well to commercial production because of the cost and lack of commercial facilities for large-scale manufacture. Therefore, efforts were undertaken to develop and evaluate other formulations that are more amenable to commercialization of an acceptable product.

Conidia harvested from liquid cultures of the non-toxigenic color mutants of *A. flavus* and *A. parasiticus* were used in the production of two different formulations for testing along with the rice formulation in environmental control plots at the National Peanut Research Laboratory. One formulation, termed Pesta, was made by an extrusion process that resulted in the encapsulation of the conidia in a wheat gluten-kaolin matrix. Pesta granules contained approximately 3×10^5 CFU/g of the *A. flavus* color mutant and 8×10^5 CFU/g of the *A. parasiticus* color mutant. The other formulation involved encapsulation of conidia in a pregelatinized corn flour, resulting in granules containing approximately 3×10^6 and 4×10^6 CFU/g of the *A. flavus* and *A parasiticus* color mutants, respectively. These formulations along with the rice (containing 1×10^6 and 3×10^6 CFU/g *A. flavus* and *A. parasiticus,* respectively) were applied to peanuts in 3 m plots consisting of 6 rows at 58 days after planting. All treatments, including non-treated controls, were replicated four times.

Aflatoxin concentrations in peanuts from control, rice, Pesta, and corn flour granule-treated plots averaged 119.8, 5.0, 30.6, and 13.8 ppb, respectively. Although these differences were not statistically significant, a trend towards reduced aflatoxin in plots to which the formulations were applied was evident. Because prior studies have shown greater aflatoxin reductions in peanuts in the second crop year after initial application of biocompetitive fungi, these studies will be repeated in 1997 to determine the longer-term effect of these formulations in combating aflatoxin contamination.

Soil populations of color mutants just prior to harvest in treated plots did not differ significantly, ranging from 56,625 CFU/g in plots treated with the corn flour granules to 80,950 CFU/g in plots treated with Pesta. Populations in rice-treated plots averaged 77,000 CFU/g. Populations of wild-type *A. flavus/parasiticus* averaged 10,475 CFU/g in control plots compared with 1222 CFU/g for Pesta-treated plots, 945 CFU/g for corn flour granule-treated plots, and 38 CFU/g for rice-treated plots. These data illustrate that the biocompetitive agents became established in plots treated with all the formulations, but final judgment concerning the effectiveness in reducing aflatoxin contamination cannot be made until the experiment has been repeated for a second year.

INCIDENCE OF *ASPERGILLUS FLAVUS* SEED INFECTION AND AFLATOXIN CONTAMINATION IN MISSISSIPPI AND TEXAS COTTON MODULES

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In two modules constructed in 1994 the number of sampling ports positive for aflatoxin increased over the length of time of storage of the modules. Analysis of aflatoxin data, however, indicated a module by time interaction. Regression analysis of mean aflatoxin concentration (mean of 16 ports) versus time was positive and significant indicating that aflatoxin concentration increased over time in the MSU I module. The relationship in MSU II was positive but not significant. We also looked at change in aflatoxin concentration over time for each port within each of the modules. There was a significant increase in aflatoxin concentration over time at 7 of the 16 ports in MSU I and at one port in MSU II. There were no instances of significant decreases in aflatoxin concentration over time. Aflatoxin was detected in all samples taken from the exterior of modules MSU I and MSU II. Levels were similar to those found within the modules and there was no significant change over time.

Yields were extremely low at the aflatoxin experimental site in 1995 with cotton available only for construction of one module. *Aspergillus flavus* seed infection and aflatoxin were lower in 1995 than 1994. Aflatoxin levels were below 1 ppb in all cases. Eleven samples of fuzzy seed (ca. 12 lb. each) were collected from the seed pipe during ginning of the module. All but one sample contained fluorescent seed. However, *A. flavus* was detected in only 3 samples of 300 seeds selected randomly from each sample and plated on MRBA. In all cases seed infection was less than 1 %.

In 1996, two modules were constructed in the Corpus Christi area of Texas and two in Yuma, Arizona, in areas with traditional high aflatoxin contamination of cotton modules. Two modules have also been constructed at Mississippi State, MS. The Mississippi State site was infested with 7.5 lbs/A of wheat upon which a toxigenic strain of *A. flavus* had been grown in an effort to increase *A. flavus* seed infection and aflatoxin contamination. Subsequent observation of wheat kernels in the field indicated prolific sporulation of *A. flavus.* Module samples from Arizona and Mississippi are currently under study.

The highest concentrations of aflatoxin found to date have been in the 1996 Texas modules. Mean aflatoxin concentration of composite samples composed of cotton meal from all sampling ports of TX I, ranged from 0 ppb at the time of moduling to 43 ppb after three weeks of storage. Regression analysis of composite samples versus time was positive and significant indicating that aflatoxin concentration increased over time in the TX I module. One external sample from TX I was 3,200 ppb. Levels of aflatoxin were lower in TX II and there was a trend toward decreasing aflatoxin concentration over time. Means of internal module temperatures were higher for TX II than for TX I. At three weeks of storage, mean internal temperature of TX I and TX II was 32 and 42.2 C, respectively. Maximum temperature for aflatoxin production has been reported as 41.5 C ± 1.5 C.

POSTER PRESENTATIONS

SOIL SURFACE POPULATIONS OF *ASPERGILLUS FLAVUS* IN YUMA COUNTY

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Aspergillus flavus isolates from Arizona can be divided into two genetically distinct strains on the basis of sclerotial morphology. On average, S strain isolates from Arizona produce much more aflatoxin than L strain isolates. To help understand factors influencing the aflatoxin producing potential of *A. flavus* populations, spatial and temporal patterns of strain S incidence were compared with patterns of total *A. flavus* population magnitude in Yuma County soils. Thirty commercial fields were selected from a cross section of cultivated land ranging from Texas Hill on the east to San Luis in the southwestern corner of the county. The nested design included several spatial scales which were named "subregions" (20-100 km apart), "areas within sub-regions" (10-15 km), "fields within areas" (1 -5 km), and "locations within fields"(150-300m). Four sub-regions contained two areas each and a fifth contained only one area. Each area consisted of three or four fields within 5 km of each other. Two locations were selected at opposite corners of each field. At each location samples were collected from three sites about 10 m apart. Population magnitude was determined by dilution plating and strain S incidence was determined by sub-culturing from the dilution plates.

Comparisons of population magnitude with strain S incidence have revealed important similarities as well as differences. Region-wide averages show that both population magnitude and strain S incidence peak during the summer. There is, however, no field by field correlation between total *A. flavus* population magnitude and S strain incidence. For example, a field high in *A. flavus* population magnitude may be low in S strain incidence or vice versa. The most important spatial scale for both population magnitude and S strain incidence is "among fields within areas" (1 to 5 km). However, there was also an important sub-regional spatial component in S strain incidence. Thus, although sub-regions did not differ in population magnitude, the Texas Hill sub-region had a significantly greater S strain incidence.

Strain S isolates were found in all sampled fields, but strain S incidence ranged from 4% to 93%. There were significant differences in strain S incidence among fields within areas in March 1995 ($p=.0195$), July 1995 ($p=.0028$), October 1995 ($p=.0009$), and March 1996 ($p=.0013$). In contrast, the correlation in strain S incidence between locations within the same field was high (overall $r=0.69$ when at least 45 isolates are characterized at each location). This means locations 150 to 300 m apart (within fields) are frequently similar in strain composition but locations 1 km to 5 km apart differ significantly. Apparently factors that vary at the sub-regional scale are influencing strain incidence. Characterization of such variables may lead to improved management of *A. flavus* community toxigenicity and aflatoxin contamination. Current work is focused on understanding the influence of crop rotation and other sub-regional variables on *A. flavus* strain composition and population magnitude.

REDUCTION OF AFLATOXIN CONTAMINATION IN TREE NUTS: ANTAGONISTIC EFFECTS OF SAPROPHYTIC YEASTS ON *ASPERGILLUS FLAVUS*

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Aflatoxins are extremely toxic and carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus.* Current research effort is directed towards the elimination of those harmful mycotoxins in food crops through biological control. Yeasts which can colonize plant surface for a very long period under dry conditions, produce extracellular polysaccharides that enhance their survivability and restrict both colonization sites and nutrient flow to other fungi on the phyllosphere. We have been investigating the potentials of yeasts as effective biocontrol agents against *A. flavus.*

There are mutants of *A. flavus* blocked in the pathway of aflatoxin biosynthesis resulting in the accumulation of various pigmented intermediates. The *nor* mutants accumulate norsolorinic acid, a bright orange colored compound, which can be easily visualized. We have applied the *nor* mutants in our screening protocol for effective antagonistic yeast strains. Each isolate of yeast was tested by scoring its inhibitory activity in spore germination, colony expansion and sporulation of the *nor* mutants. This visual assay provides a useful tool to distinguish several biocontrol mechanisms of yeasts in preventing aflatoxin production by *A. flavus.*

We have isolated a number of yeasts from the surface of pistachio, almond, walnut, avocado, apple and lemon for the screening of an effective antagonist. Several yeast strains can confine the spreading of *nor* mutants and reduce the red orange color formation, which is an indication of preventing aflatoxin production. We have constructed a double agar plates assay system to determine the effect of yeast volatiles on aflatoxin formation. Volatiles from yeast strains W4 and CS5 almost completely inhibited the accumulation of norsolorinic acid and the fungal colonies are white. The results indicated that volatiles of yeasts could effectively prevent aflatoxin production. The volatile constituents are being analyzed by combined gas chromatography and mass spectroscopy. Certain strains of yeasts attached to the fungal hyphae and reduced the growth and sporulation of *A. flavus.*

HPLC DETERMINATION OF MAJOR SECONDARY METABOLITES PRODUCED BY *ASPERGILLUS* SECTION *FLAVI* ON LIQUID MEDIUM

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Secondary metabolites of *Aspergillus* section Flavi, such as aflatoxins, cyclopiazonic acid (CPA) and *O*-methylsterigmatocystin (OMST), are known to elicit a toxic response in vertebrate animals. These metabolites represent diverse chemical classes which makes their simultaneous determination extremely difficult. The existing chemical methods for quantitative determination of secondary metabolites produced by *Aspergillus* section *Flavi* do not provide needed simplicity, accuracy and reliability. The purpose of this work was to develop a simple, accurate and fast method for simultaneous quantitation of major metabolites produced by *Aspergillus* section *Flavi* on a liquid medium. An HPLC method for simultaneous determination of major secondary metabolites, including CPA, OMST, and the versicolorins, produced by *Aspergillus* section *Flavi* (*A. flavus, A. parasiticus, and A. tamarii*) on a liquid medium has been developed. The metabolites were extracted with chloroform and quantified without any cleanup procedure for means of normal-phase ion-pair partition HPLC on silica gel using *n*-Heptane - 2-Propanol - Water - Tetrabutylammonium hydroxide (2560:1120:32:8, v/v's) as a mobile phase. Recoveries of CPA and OMST from the fungal cultures spiked at 10 ppm were 98.90±3.27 and 95.92±5.27, respectively. The limit of detection for pure standards was 0.25 μg/ml for CPA (at 280 nm) and 0.30 μg/ml for OMST (at 310 nm). The chromatographic system was capable of simultaneous separation of both acidic (CPA, kojic acid, aspergillic acid, etc.) and neutral (OMST, ergosterol, etc.) fungal metabolites within 10 minutes. This was possible due to the ion-pair partition mechanism of the separation. The mobile phase was UV-transparent up to 210 nm, which allowed the use of a diode array detector to confirm the identity of fungal metabolites. Due to the high concentrations of the metabolites, no cleanup procedure for the chloroform extracts was required. This method is useful for the study of toxin-production and chemical taxonomy within *Aspergillus* section *Flavi* as well as the study of biosynthetic pathways of aflatoxins.

CROP RESISTANCE - CONVENTIONAL BREEDING

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Identification of Aflatoxin Resistance Traits and Incorporation into Commercial Germplasm

PANEL MEMBERS: R. Brown (Chair), T. Gradziel, C. Holbrook, D. White, T. Rocheford, C. Martinson, D. Wicklow, N. Widstrom, and D. Wilson

There was general agreement by panel members that significant progress had been made towards the development of germplasm (corn, peanut and almond) resistant to *Aspergillus flavus* infection/aflatoxin contamination. This progress can best be demonstrated not only by assessing how close we are to the arrival of a "final product" (commercially viable resistant cultivars), but also by evaluating the development of the various "products" (technologies) that of necessity precede the "final products." The identification of resistant corn, peanut and almond germplasm owes a great deal to improvements in the methods of screening for resistance. These methods include the pinboard inoculation technique, kernel screening laboratory assay (KSA), and an aflatoxin-inhibition nondestructive bioassay. Easy screening tools also included: 1) the use of norsolorinic acid (NOR-an easily visualized red pigment associated with the aflatoxin biosynthetic process) producing *A. parasiticus* mutants for field-screening corn, 2) assessment of peanut plant leaf temperature and visual stress ratings in cultivar selection, and 3) use of an *A. flavus*-glucuronidase (GUS) gene-containing transformants in the assessment of fungal infection; GUS is a bacterial enzyme yielding unique colored or fluorescent products as indicators for "tracking" and quantifying growth of the fungus containing the GUS gene.

Progress towards production of resistant cultivars has been demonstrated by the development of almond cultivars with both fungal and insect vector resistance. It has also been demonstrated by the association of both specific corn kernel proteins with resistance, and the investigation of the association of chemical constituents of corn kernel wax with resistance. Mapping and breeding studies also are being conducted using corn kernel wax as a resistance marker.

In certain corn genotypes, we have gained an understanding of the inheritance of resistance and have found that several sources of resistance have sufficient dominance that resistance may only need be present in one of two parents of a hybrid. The location of resistance genes in these corn lines is also being determined, and with some sources of resistance, genes are at different loci in the corn genome indicating multiple genes involved in aflatoxin resistance. This means that it may be possible to pyramid genes achieve high levels of resistance, and yet exclude undesirable qualities. Avoiding undesirable traits is of great importance since resistant cultivars, to be commercially useful, must possess other agronomically acceptable traits. Resistant almond breeding lines demonstrating more commercially acceptable qualities continue to show promise. Peanut lines with resistance have been entered into a hybridization program with agronomically acceptable lines. Progress also is being made in the transfer of resistance to B73 and Mo17 elite corn germplasm. These advancements in the understanding and the manipulation of resistance mechanisms may provide the basis for the development of cultivars with different sources of resistance, a prospect necessitated by the potential of pathogens to mutant and negate the usefulness of certain resistance genes.

During the panel discussion, there were questions concerning the ability to distinguish the red color of corn kernels infected by the *A. flavus* NOR mutant. Positive results have been obtained using this fungus in tests both in Georgia and in Weslaco, Texas. However, this method might be ineffective if used on many tropical or ornamental collections for which segregation of red, brown or purple aleurone and/or pericarp was occurring.

Despite the progress made in the last few years towards the development of commercially acceptable germplasm with resistance to *A. flavus*/aflatoxin contamination, the panel discussion reflected a need to gain an even clearer understanding of the fungal infection process in host tissues which lead to aflatoxin production.

PLATFORM PRESENTATIONS

PROGRESS IN INTEGRATING FUNGAL PATHOGEN AND INSECT VECTOR RESISTANCE FOR PREHARVEST AFLATOXIN CONTROL IN ALMOND

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The domestic and export almond markets presently demand zero to very low levels of aflatoxin contamination. While aflatoxin contamination in almond is relatively infrequent, when present it can occur at very high concentrations, making it a serious health and economic problem. To achieve the necessary high levels of control, we are attempting to integrate multiple genetic mechanisms for control of *Aspergillus spp.* fungi as well as Navel orangeworm *(Paramyelois transitella* Walk.) which appears important for initial fungal infection.

1996 project goals include: large scale field testing of resistant cultivars and advanced breeding selections, development of procedures for genetically engineering the major almond cultivar *Nonpareil,* development of procedures for synthesizing chimeric *Nonpareil* almond with resistant epidermis, and incorporating the most promising resistance into next generation cultivars.

Resistance to Navel orangeworm infestations has previously been identified in a well-sealed endocarp, and in either antibiosis or nonpreference in the hull and/or seed tissue. Regional trials of the resistant cultivar *Mission* and advanced breeding lines have been established at all major California production areas with the first commercial harvest occurring this season. In all test areas, nut damage from insect infestation was as high as 40% in the susceptible cultivar *Nonpareil* yet consistently remained well below 1% in *Mission* and the selected breeding lines. X-Ray and microscopic analysis of over 10,000 *Mission* nuts from highly infested orchards suggests that when resistance does breakdown, it is through failure of the shell suture seal rather than other, less understood pathways. Thus the integrated resistance strategy appears effective and stable following one season of regional testing. Results from controlled insect feeding studies showed effective antibiosis of *Mission* hulls at the time of hull split, though this response could not be consistently detected when dry hulls were used in the feeding study. Fungal contamination of hulls was a problem when fresh material was utilized.

Nonpareil leaf explants can now be consistently transformed with stable expression of tile reporter genes used. Regeneration of shoots from these explants has been increased from a rate of approximately 12% to up to 28% using an MS regeneration media containing IBA (0.5 mg/L), BA (2-3 mg/L), and TDZ (1-3 mg/L). No transformants have been regenerated to date, however, and possible restriction to regeneration are being investigated. Regeneration of transgenics has been successful using similar methods for the *Hansen* almond-peach hybrid.

Synthetic periclinal chimeras consisting of *Nonpareil* tissue with a peach epidermis have been achieved through apical micro-grafting. This approach may offer a powerful and rapid strategy for incorporating complex resistance to even recalcitrant vegetatively propagated cultivars.

THE SEARCH FOR RESISTANCE TO PREHARVEST AFLATOXIN CONTAMINATION IN PEANUT

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Preharvest aflatoxin contamination (PAC) is one of the most serious challenges facing the U.S. peanut industry. The objectives of this research program are to identify sources of resistance to PAC and to use these sources to develop resistant peanut cultivars. To facilitate the identification of resistant genes, a core collection was selected to represent the entire germplasm collection for peanut.

All accessions in the peanut core collection were examined in a preliminary screen using five replications at either Tifton, Georgia, or Yuma, Arizona. Genotypes that had low contamination levels in the preliminary screen were then examined in additional tests. Thirteen core accessions have showed at least a 50% reduction in PAC for three years of testing. An additional 27 core accession have showed at least a 50% reduction in PAC for two years of testing.

Research was also conducted to examine the relationship between resistance to other fungi and PAC and between altered fatty composition and PAC. Peanut genotypes with resistance to leaf spot and/or white mold, did exhibit a reduced level of colonization by *Aspergillus* and did not exhibit a reduced level of PAC. Peanut genotypes with reduced linoleic acid composition have not exhibited a consistent decrease in PAC.

We have previously documented drought tolerance and reduced levels of PAC in six peanut plant introductions with extensive root systems. A significant positive correlation between leaf temperature and PAC and between visual stress rating and PAC indicates that these measurements may be valuable indirect selection tools. Aflatoxin contamination is an expensive trait to measure. The use of leaf temperature or visual stress ratings for preliminary screening of breeding populations for resistance to PAC would greatly reduce the expense of developing resistant cultivars.

Many of the lines which we have identified as having lower PAC have less than acceptable agronomic characteristics. These lines have been entered into a hybridization program to combine resistance to PAC with acceptable agronomic performance. We are also intermating the resistance lines. The objective of this is to combine different genes for resistance to produce genotypes with even higher resistance to PAC.

FURTHER PROGRESS AND BREEDING FOR AND MAPPING GENES FOR RESISTANCE TO *ASPERGILLUS FLAVUS* AND AFLATOXINS IN CORN

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Research at the University of Illinois is divided into four interrelated components including: 1) identification of resistance; 2) determining the inheritance of resistance; 3) molecular marker mapping of genes for resistance; and 4) crossing resistance into B73 and/or Mo17 related inbreds.

Identification of Resistance. We have screened more than 1200 corn inbreds as Fl crosses with susceptible inbreds Mo17 and/or B73 for resistance to *Aspergillus* ear rot and aflatoxin production. All screening has been done using artificial inoculation (2). We have identified 13 inbreds that are highly resistant in Fl combination, resistant as inbreds per se, and many of which have been resistant in studies by others (1,3,5,6).

Inheritance of Resistance. We have studied the inheritance of resistance to Aspergillus ear rot with nine inbred lines (4) and resistance to aflatoxin production in three of the nine. The three inbreds for which we have the most data include LB31,75-Rool, and Tex 6. Generation mean analysis indicates high estimates of dominance genetic effects with several of the sources of resistance.

During the last two years, we have concentrated our efforts on understanding the inheritance of resistance of Tex 6 x Mol7 and Tex 6 x B73. Frequency distribution of aflatoxin content of ears of F3 families, backcross susceptible-self families and backcross resistance-self families of Tex 6 x Mol7 and Tex 6 x B73 are highly skewed towards the resistant parent, indicative of genetic dominance. The resistance from Tex 6 is of a high level and highly heritable. Low correlations between ear rot rating and aflatoxin indicate that different genes may control resistance to either ear rot or aflatoxin production.

Evaluation of a series of F1 crosses of our best sources of resistance with one another and with Mo17 and B73 in 1995 supported the earlier finding of Tex 6 as one of the best sources of resistance. The best five F1s for ear rot resistance had Tex 6 as a parent and the best four F1s for aflatoxin levels had Tex 6 as a parent (8). This study was repeated in 1996.

Mapping genes for Resistance. Three inbreds (LB31, 75-Rool, and Tex 6) have been studied utilizing RFLP analysis to identify chromosomal regions associated with the resistance to Aspergillus ear rot and inhibition of aflatoxin production. Most interestingly, some chromosome regions have been associated with resistance to both aflatoxin production and resistance to Aspergillus ear rot while others are associated with only resistance to ear rot or resistance to production of aflatoxin (7). Finding chromosome regions from more than one source of resistance that are associated with resistance provides independent supportive evidence of the existence of resistance genes at certain locations. Other regions associated with resistance to ear rot or aflatoxin production are not common in the different mapping populations suggesting that there also are different genes for resistance from the different

sources of resistance. RFLP mapping supports our findings of low correlations between Aspergillus ear rot and aflatoxin production. The possibility of different mechanisms for ear rot resistance and for resistance to aflatoxin production also is supported by other research (5,6). Some of the chromosomal regions associated with resistance are in the same chromosomal regions that have clusters of disease and insect resistance genes (9).

Breeding for Disease Resistance. We have made progress in transferring resistance from the inbred LB31 into B73 types while maintaining yield. Yield of several of our S-3 to S-5 lines in test cross combination with Mo17 types produce hybrids resistant to Aspergillus ear rot and aflatoxin production that have yield similar to susceptible commercially used hybrids. The inbred 75-Rool does not appear to be a good source of resistance in a breeding program. In general, we have been able to transfer resistance, however, yield performance in hybrids and other agronomic characteristics are poor. Preliminary evidence suggests that Tex 6 can improve both B73 and Mo17 types, however, we are currently concentrating on utilizing Tex 6 to improve Mo17 types. Our ultimate goal is to be able to have a B73 x Mol7 type hybrid with resistance from LB31 on the B73 side of the cross and resistance from Tex 6 on the Mol7 side of the cross. We will then have genes from two distinctly different sources of resistance in the hybrid. It is important to understand that if resistant lines are to be used by commercial seed corn companies either directly or as sources of resistance they must have good agronomic characteristics.

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DEVELOPMENT OF INBREDS WITH ENHANCED RESISTANCE TO AFLATOXIN SYNTHESIS THROUGH MUTATION BREEDING

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Resistance to aflatoxin production is a desired trait in those crops where aflatoxin has become a problem. In corn we have found induced mutants in inbred lines B73 and A632 that have hexane extractable components of the seed which are inhibitory to aflatoxin synthesis by *Aspergillus flavus*. A bioassay procedure was used to select for mutants in mutagenized B73 and A632 (source inbred lines for many of the current corn belt inbreds and hybrids) that produce high levels of inhibitors of aflatoxin synthesis. The progeny from four M3 families of A632 and two M3 families of B73 have been developed by inbreeding to the M4, M5, and in 1996 to the M6 stage. The M3, M4, and M5 families have been selfed and crossed to non-mutagenized A632 and B73 inbred lines. The M3 families were also crossed with Mo17 to develop hybrids for evaluation of agronomic traits. The M3 families were screened for single seeds with high resistance to aflatoxin synthesis, which was a rare event. When a resistant seed was found, the residual seed from that family was grown out and selfed to form M4 progeny. Ten to 14 seeds from each M4 ear were bioassayed and we found that most of the ears did not have kernels with aflatoxin inhibitory properties in hexane extracts from individual seeds. When two or more seeds were found to be highly inhibitory, residual seed from those families were planted to be selfed for the M5 generation. During the screening of M4 seed, it became apparent that an intermediate level of inhibition was being observed from extracts from some of the seeds. Crosses of M3 progeny with the non-mutagenized respective inbred line usually yielded seeds with no increased inhibition of aflatoxin synthesis and if any inhibition was expressed it was of the intermediate type. The frequency of finding progeny seed with high levels of aflatoxin inhibition increased greatly in the M5 generation. The M4 families used to develop the M5 generation had at least 20% of the kernels expressing high levels of aflatoxin inhibition, therefore nearly all M5 progeny expressed some level of inhibition. It was common to find M5 ears with over 50% of the kernels with high levels of inhibition of aflatoxin synthesis and the balance of the kernels being intermediate in reaction. None of the M5 ears had 100% of the kernels with high levels of inhibition of aflatoxin synthesis. There must be several genes involved, and one wonders if the mutant genes are unstable, or difficult to accumulate in the progeny.

Seed produced in 1996 will be M6 progeny, crosses to non-mutagenized A632 and B73 and selfs of prior crosses of mutagenized x non-mutagenized A632 and B73. This will allow for a better grasp on the inheritance of the trait. Many of the M6 ears should have a high frequency of kernels with high levels of any putative inhibitor of aflatoxin synthesis. There should be adequate M6 seed to allow for analyses for compounds that may be responsible for the increased inhibition of aflatoxin synthesis.

Mo17 x B73 and Mo17 x A632 hybrids made from both mutagenized and non-mutagenized B73 and A632 inbreds were compared for agronomic traits in 1996. Within a hybrid grouping we found no differences in tassel branch number, ear number, or ear height among any of the crosses within a hybrid grouping. Some Mol7 x B73 (mutagenized) crosses were either taller or shorter than the normal Mo17 x B73. Yield data will also be collected.

The elite mutagenized B73 and A632 germplasm being used in this research was developed originally by Allen D. Wright when he was associated with the USDA-ARS at Iowa St. Univ.

ADVANCES IN IDENTIFYING AND CHARACTERIZING MAIZE KERNEL BIOCHEMICAL RESISTANCE TRAITS FOR INCORPORATION INTO A COMMERCIAL BREEDING PROGRAM

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It was determined that GT-MAS:gk and MI82, maize genotypes with demonstrated resistance against *Aspergillus flavus* infection and aflatoxin production, supported significantly less growth of *F. moniliforme* than did susceptible kernels. The resistance mechanisms involved in these genotypes may have broad antifungal applications. The wax layer in GT-MAS:gk kernels may be a source of mechanical resistance worth incorporating into commercial germplasm. The possibility also exists that GT-MAS:gk wax conveys chemical resistance against *A. flavus* infection. Qualitative wax studies are presently being pursued. The results of a study of 2 resistant and 2 susceptible maize genotypes show that differences in protein profiles and in vitro antifungal bioactivity exists between the resistant and susceptible lines. In dry kernels, the concentration of the active forms of RIP (ribosome-inactivating protein) appear higher in resistant than in susceptible lines. Thus, the possibility exists that preformed chemicals are responsible for some of the resistance observed in GT-MAS:gk and Mp420. Investigations of germinating kernels demonstrated that zeamatin, a known antifungal protein, is induced to higher concentrations by germination in both resistant and susceptible genotypes. Also, RIP changed from inactive to active forms with germination in all tested lines. These changes may be related to the germination-induced resistance previously observed in susceptible maize lines. Through conventional breeding, gene mapping strategies, GUS technology and laboratory screening, the inheritance of resistant mechanisms operable in these maize genotypes and in other germplasm may be better understood, and several types of resistance may be combined into a single line for incorporation into commercial germplasm.

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AFLATOXIN AND ERGOSTEROL IN BGYF KERNELS FROM RESISTANCE TRIALS

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Commercial corn hybrids and inbreds showing numerous undamaged BGYF kernels were identified in aflatoxin resistance trials conducted at Mt. Vernon, IN (1995). These BGYF kernels offer proof that *Aspergillus flavus* has gained entry to the seed proper, infected the germ, and produced kojic acid. Evidence from histopathological studies had earlier shown that the immediate pathway of *A. flavus* entry is through random tears in the seed coat over the embryo. Seed coat tearing appears to be under genetic control. Our data reveals that B73 and OH43 inbreds commonly show a high frequency of BGYF kernels, while Mo17 inbreds do not. Furthermore, an elite inbred Mo17/OH43 produced numerous undamaged BGYF kernels, suggesting perhaps that OH43 contributed this susceptibility to seed coat tearing. RFLP analyses might be used to identify genetic markers for chromosomal regions associated with seed coat tearing, enabling breeders to eliminate this trait from commercial hybrids.

We contrasted aflatoxin and ergosterol levels of BGYF kernels from resistance trials as a strategy for identifying sources of chemical and biochemical resistance to *A. flavus* infection and/or aflatoxin biosynthesis. Ergosterol values enable us to estimate fungal biomass and extent of kernel infection. BGYF kernels were removed from each grain sample (harvested row), milled, and analyzed for aflatoxin (ppb) and ergosterol (ppm). There was substantial variation in the amount of aflatoxin accumulation in BGYF kernels from hybrids (e.g., 90-4800 ppb) and inbreds (e.g., 12-1294 ppb). Likewise, estimated actual ergosterol values showed substantial variation among corn varieties (hybrids = 1.3-21 ppm; inbreds = 1.4-281 ppm). This is further evidence to suggest that some corn varieties may differ in their internal resistance to pre-harvest *A. flavus* infection and aflatoxin contamination.

With most hybrids, removal of the aflatoxin-contaminated BGYF kernels afforded grain samples with < 20 ppb aflatoxin. These results suggest that the elimination of seed coat tearing in commercial hybrids could have an immediate, positive impact in eliminating aflatoxin from preharvest corn. Our goal is to produce hybrids that combine resistance to seed coat tearing with germ and endosperm resistance to *A. flavus* infection and aflatoxin biosynthesis.

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*Ergosterol analyses were performed by Robert A. Norton, Mycotoxin Research Unit, NCAUR. In 1996, we continued our cooperative work with Cargill Hybrid Seeds, Mt. Vernon, Indiana.

SCREENING FOR RESISTANCE IN CORN TO AFLATOXIN CONTAMINATION USING AN *ASPERGILLUS PARASITICUS* MUTANT (ATCC 24690)

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Methods for determining resistance in corn to kernel infection by *Aspergillus flavus* or aflatoxin contamination have been costly due to the labor and/or materials required for testing. Cost effective procedures are needed to facilitate mass screening of maize germplasm. We inoculated kernels of several maize hybrids and plant introductions with an aflatoxin producing mutant of *A. parasiticus* that also produces norsolorinic acid resulting in a reddening of the kernel aleurone layer when infection occurs. Eight hybrids were evaluated in 1994 and 1995. Significant differences among hybrids occurred for the square root of the number of red kernels/sample and log ng g^{-1} aflatoxin in both years. The correlation between aflatoxin and red kernels was highly significant in 1994, but nonsignificant in 1995. The discrepancy was apparently due to a greater level of infection by naturally-occurring *A. flavus* in 1995 (12.0%) than in 1994 (2.5%) that changed the relative levels of aflatoxin for some hybrids. Fifty-eight plant introductions were evaluated in 1995 and a 15-entry subset of the 58 were evaluated again in 1996. Entry means differed significantly for red kernel traits and aflatoxin contamination in 1995. Only number of red kernels per plot and number of red kernels per kg of grain were significant in 1996. Correlations of entry means indicated that red kernel traits are effective indicators of resistance to aflatoxin when large numbers of entries are being evaluated or when infection by naturally occurring *Aspergillus* isolates is low. Use of the *A. parasiticus* aleurone marker should be effective in identifying the bulk of susceptible genotypes during the initial stages of mass screening, thus significantly reducing the cost of this phase of screening. Only the better performing entries then need be evaluated in the next phase of screening by precise but more expensive methods.

PROGRESS ON USING THE RED *ASPERGILLUS PARASITICUS* MUTANT IN DETERMINING RESISTANCE IN CORN

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The norsolorinic acid (NOR) producing *Aspergillus parasiticus* mutant (ATTC 24690) has been used for the last three years to develop a screening method for resistance to preharvest aflatoxin contamination of corn. When corn is infected by this mutant, it produces NOR and aflatoxin. The NOR can be visually observed. In 1994 and 1995, there was a significant correlation between aflatoxin content and number of red kernels; in 1996 this was not observed. Forty days after full silk very few red kernels were observed, but by harvest the NOR had accumulated and red kernels were observable. The two hybrid test using F 4507A as the susceptible hybrid and MP313ExMAS:gk as the moderately resistant hybrid was designed to find if the differences in red kernels and aflatoxin would be repeatable over years. In 1994, there was a significant difference in red kernels and aflatoxin content between hybrids. In 1995 and 1996, the differences were consistent but not significantly different. The incidence of natural *Aspergillus flavus* group seemed to interact and influence the incidence of *A. parasiticus* and red kernels. There was a negative correlation between the incidence of *A. flavus* group and *A. parasiticus*. Weather seems to be an important determinant in all aflatoxin studies. Interpretation of our results suggests that weather influences not only the host plant but the native microflora.

ELIMINATION OF AN ARTIFACTUAL BLUE COLOR POTENTIALLY INTERFERING WITH THE β- GLUCURONIDASE (GUS) HISTOCHEMICAL DETECTION OF *ASPERGILLUS FLAVUS* COLONIZATION OF MAIZE KERNELS

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Aspergillus flavus strains containing reporter genes are being utilized to assess mode of fungal entry and invasion in plants and host plant resistance mechanisms. Cotton flowers in a greenhouse environment were nectary-inoculated with an *Aspergillus flavus* strain (GAP 2-4) transformed with the *Escherichia coli* β-D-glucuronidase (GUS) reporter gene linked to an *A. flavus* β-tubulin gene promoter. At maturity, seeds from inoculated bolls, were plated onto agar medium in an attempt to recover the inoculated fungal transformant. However, a wild-type *A. flavus* strain (SRRC 1000A) used to inoculate nearby bolls was recovered from a few GAP 2-4 inoculated plants, and when plates containing fungi plated on agar media were assayed histochemically for GUS activity (blue color indicates GUS expression), agar plugs containing strain 1000A showed blue color, normally indicative of GUS activity. These results elucidated the need to reduce potential false positive results in the interpretation of GUS histochemical assays. To accomplish this, several quantitative (colorimetric) and quantitative experiments, testing literature recommendations for eliminating false blue positives and employing a variety of *A. flavus* isolates, were performed on fungal cultures and on maize kernels. Results indicate that at pH 7.0, the pH recommended for GUS activity assays, only GAP 2-4 turns blue either in culture or in kernel internal tissues. Also, quantitative analysis revealed GUS activity only in strains known to contain a GUS gene. Subpericarp blue staining, which occurred on some kernels inoculated with strain 1000A, was eliminated by heat-treating seed prior to assaying for GUS expression. GUS detection buffer pH and strength appear to be the most critical areas requiring attention to insure a clear window for the detection of GUS expression. Literature recommendations proved very useful in eliminating artifactual blue staining.

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INDUCTION OF PROTEINS AND RESISTANCE TO *ASPERGILLUS FLAVUS* DURING CORN KERNEL GERMINATION

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Pathogenesis-related (PR) proteins in corn could provide corn breeders with valuable selectable markers for incorporation of resistance into corn to *Aspergillus flavus*. For many plant-pathogen systems, inducible PR proteins have been well documented. In corn, these PR-proteins including hydrolases, ribosome-inactivating-proteins (RIP), and zeamatin, have been studied. Our previous studies demonstrated that preincubation of kernels at 100% relative humidity, prior to fungal inoculation, induced resistance to aflatoxin production in susceptible genotypes. The objectives of this presentation were to study the protein inductions from preincubation, germination, and infection by *A. flavus*, and to examine the bioactivity of extracts from kernels subjected to germination. Four corn genotypes were included in this study: 2 resistant (GT-MAS:gk and MP420) and 2 susceptible (Pioneer 3154 and Deltapine G-4666). SDS-PAGE resolved 5 protein bands induced to a higher concentration in germinated kernels than in non-germinated kernels. Three bands were below 24 kDa in all 4 genotypes. However, protein patterns of resistant corn kernels preincubated and then infected with *A. flavus* contained an extra band with a molecular weight of 20 kDa that was absent in the two susceptible corn genotypes. Western blot analysis revealed that one protein was reacting with 22 kDa zeamatin antibody and this protein was accumulated to a higher concentration in germinated kernels. Using antibody against RIP indicated that RIP primarily existed in the pre-RIP form in non-germinated kernels; however, in germinated kernels RIP existed essentially in the activated forms. Antifungal bioassays of germinated kernel extracts of all 4 genotypes exhibited strong antifungal activity. The two susceptible genotypes caused even bigger inhibition zones. This study provides evidence of protein induction in corn kernels and the findings here may be related to previous observations of germination-induced resistance in susceptible corn genotypes.

PROTEIN PROFILES AND ANTIFUNGAL ACTIVITIES OF CORN KERNEL EXTRACTS FROM GENOTYPES RESISTANT AND SUSCEPTIBLE TO *ASPERGILLUS FLAVUS*

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In modern times, mycotoxin contamination of food and feed as a threatening factor to human health has inspired different research approaches world-wide in an effort to reduce or eliminate this problem. Our interests are to understand the biochemical and molecular basis governing corn genotypical differences in aflatoxin production. In this study, we included two resistant genotypes, GT-MAS:gk and MP420, and two susceptible genotypes, Pioneer 3154 and Deltapine G-4666, with the objective of examining the pre-formed proteins and the *in vitro* antifungal bio-activity of each genotype. Proteins from corn kernel extracts of the two groups were analyzed with SDS-PAGE, native PAGE, and 2-D. Differences in protein profiles between the resistant and susceptible groups were found. Some bands present in resistant genotypes were absent in susceptible ones and vice versa. Some bands on SDS-PAGE or native PAGE were intense in the resistant group and minor in the susceptible group. Antifungal bioassays of kernel extracts from resistant and susceptible genotypes also showed differences between these two groups; resistant kernel extracts produced larger zones of inhibition against fungal growth than did susceptible kernel extracts. These differences in protein profiles between resistant and susceptible genotypes may be related to previously observed differences in resistance to aflatoxin accumulation between the two corn groups. These differences also support the hypothesis that an internal resistance mechanism, possibly pre-formed chemicals (proteins), may be active in kernels of some corn genotypes. The data indicates that there may be a biochemical and molecular basis to the resistance observed in resistant genotypes, and thus, the possibility exists for the improvement of this resistance through conventional breeding and/or genetic engineering.

EVALUATION OF CORN GERMPLASM FOR RESISTANCE TO AFLATOXIN PRODUCTION

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Infection of corn with *Aspergillus flavus* and the subsequent accumulation of aflatoxin in the grain is a sporadic problem in the Corn Belt, but a chronic problem in the South. Drought stress, high temperatures, and insect damage exacerbate the problem. An extremely potent carcinogen, aflatoxin affects humans and livestock. Contamination of corn grain also limits its marketability. Grain with levels of aflatoxin exceeding 20 ppb is banned from interstate commerce. Some countries adhere to even more stringent standards for imported grain.

A potentially effective and highly desirable method of reducing aflatoxin in grain is the use of genetically resistant hybrids. Before such hybrids can be made available to farmers, however, germplasm with resistance to aflatoxin accumulation must be identified. Devising reliable inoculation techniques that produce uniform fungal infections without overwhelming the plant's resistance mechanisms and choosing appropriate methods for quantifying kernel infection and aflatoxin accumulation have provided challenges.

Open pollinated varieties, exotic germplasm crosses, and test crosses were evaluated for preharvest accumulation of aflatoxin in experiments conducted at Mississippi State in 1995 and 1996. In all experiments, the top ear of each plant in a plot was inoculated 7 days after silks had emerged from 50% of the plants using the side needle technique with a 3.4 ml suspension containing 3 x 10^8 *A. flavus* conidia. Ears were harvested 63 days after midsilk. Aflatoxin contamination was determined using the Vicam Aflatest (Watertown, MA).

Aflatoxin accumulation levels among 44 open pollinated varieties ranged from 27 ppb for Wafts Special Yellow (PI 540765) to 2168 for Flint (PI 540779). Other varieties exhibiting relatively low levels of aflatoxin included John A. Griggs (PI 540782) and J.N. Price (PI 540769). Among 30 germplasm crosses (50% exotic) obtained through the Germplasm Enhancement Maize (GEM) Project, three Brazilian germplasm crosses (BR51501:Sl1A, BR52060:SO2, BR52051:Sl7) exhibited the lowest levels of aflatoxin. Among a group of lines crossed onto Va35, Tex 6, Mp420, and Mp3l3E performed well. Two breeding lines, Mp72:299 and Mp8O:04, also exhibited low levels of aflatoxin when crossed with Va35. Mp72:299, Mp3l3E, and Tex 6 performed well in test crosses with Mol18W.

The open pollinated varieties and exotic germplasm that exhibited the lowest levels of aflatoxin in this investigation may be useful as sources of resistance. The evaluation of inbred lines and breeding lines *per se* is frequently difficult because of low grain yield and lack of vigor. It appears that evaluating the lines in test crosses was relatively effective in identifying the more resistant lines.

CROP RESISTANCE - GENETIC ENGINEERING

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Summary of the Crop Resistance-Genetic Engineering Poster Platform Session.

PANEL MEMBERS: Peggy Ozias-Akins (Chair), Jeff Cary, Mike Hasegawa, Gail McGranahan, Caryl Chlan, and Arthur Weissinger.

SUMMARY OF PRESENTATIONS: A brief overview of each of the participants poster presentations was given by Dr. Jeff Cary, USDA-ARS, Southern Regional Research Center, followed by a more detailed presentation by each presenter. The overview touched on the research of each scientist as it relates to the various experimental phases required to identify, transform, and express antifungal genes in host crops. The following summarizes each investigators work:

Mike Hasegawa isolated and purified two low molecular weight proteins from wheat that exhibited antifungal activity against *Aspergillus flavus*. A 13 kD protein identified as a PR-4 like protein inhibited *A. flavus* spore germination (ED_{50}) at concentrations of 6.5 mg/ml. An 8 kD protein identified as a wheat trypsin inhibitor gave an ED_{50} at a concentration of about 7 mg/ml. Dr. Hasegawa's group is currently examining the synergistic effects of these two proteins against *A. flavus* and are also attempting to obtain cDNAs for both.

Jeff Cary discussed the construction of a number of binary vectors based on the expression of the Demeter antifungal peptide D4E1. In addition, he presented data detailing the resistance of the D4E1 peptide to both fungal and cotton proteolytic degradation. Another protein, haloperoxidase, was discussed as a potential antifungal gene for expression in plants.

Gail McGranahan described the ability of walnut somatic embryos transformed with barley or nettle lectin, or the Demeter peptide D5D to significantly reduce *A. flavus* sporulation. In addition, embryo lines expressing the hevein protein and snowdrop lectin inhibited sporulation somewhat, but not at statistically significant levels. Electron microscopy work revealed morphological changes in fungal hyphae growing in the presence of the lectins and hevein. Caryl Chlan (University of Southwestern Louisiana) - Discussed improvements to *Agrobacterium*-mediated cotton transformation protocols. Parameters such as tissue age, light, selective pressure, and *Agrobacterium* growth phase all were important to the successful transformation and regeneration of cotton hypocotyl, cotyledon, and meristematic tissues.

Peggy Ozias-Akins discussed the potential of the soybean vegetative storage protein (vsp) promoter as a means of regulating expression of antifungal genes. Studies with vsp-GUS constructs in peanut showed that GUS expression was developmentally controlled being much higher in stems and leaves than in root. GUS activity could also be increased by exposure to jasmonate demonstrating chemical modulation of the vsp-promoter. Expression was also high in peanut pods in R1 plants. Further studies on pod expression in R2 progeny are being conducted.

Arthur Weissinger described the efficacy of tobacco plants transformed with Demeter antifungal peptide D5C. Pathogenicity assays using tobacco transformed with D5C constructs showed

significant inhibition of Cercospora and Phytophthora infection. Also described improved methods for transformation of peanut.

SUMMARY OF PANEL DISCUSSION: The panel discussion on genetic engineering of plants for resistance against aflatoxin producing fungi and overcoming critical technological barriers has focused in previous years mainly on the plant transformation systems themselves. The transformation systems for cotton, walnut, and peanut are more efficient and at a level of usefulness that, as a result, the 1996 workshop became more focused on the effectiveness of antifungal genes that could be and are being introduced into these crops vulnerable to contamination by aflatoxin. The potential efficacy of several genes against aflatoxin contamination was discussed, including those encoding antifungal peptides, either synthetic or bacterially-derived, and small proteins, a few of which are still being characterized for their effectiveness as antifungal agents. *In vitro* (in the "test tube") assays have indicated that some of these peptide/protein products are extremely effective in inhibiting the growth of *Aspergillus flavus*, an aflatoxin producing fungus. Panelists all agree, however, that the best test for efficacy of antifungal agents would be to transform the genes encoding the antifungal peptide/protein into the crop plant which could rapidly provide leaf material for *in vitro* or *in vivo* assays, direct assay of transgenic callus, or direct *in vitro* testing in fungal culture with the plant-synthesized gene product.

The gene encoding the synthetic peptide, D4E1, is of interest since this peptide demonstrated 100% lethality of germinated *A. flavus* conidia at concentrations as low as 10-15 μM (similar to results observed using D5C, a relative of D4E1) in "test tube" type assays. Of extreme interest is the fact that the gene for D5C (the gene product of which inhibits *A. flavus in vitro*) showed high potency when expressed in a model plant system (tobacco) against other fungal pathogens which attack the foliage. These important experiments demonstrated that these small synthetic peptide genes are being expressed authentically and at antifungal levels in higher plants, and thus, should be effective against aflatoxin producing fungi that can attack cotton, walnut and peanut. D5C and D4E1 have been transformed into these crops and tests have begun to assess the ability of these peptide genes to prevent the attack of crops by aflatoxin producing fungi. Other peptides of interest included a novel, small molecular weight peptide from *Bacillus* which showed high potency against *A. flavus*; a PR-4-like protein and a trypsin inhibitor also demonstrated antifungal activity against *A. flavus* and the genes encoding these proteins/ peptides will soon be available for plant transformation. The importance of driving expression of these antifungal genes under defined environmental or developmental stages by using inducible promoters also was discussed.

PLATFORM PRESENTATIONS

GENETIC ENGINEERING OF COTTON TO ENHANCE RESISTANCE TO *ASPERGILLUS FLAVUS*

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We have been developing genetic methods to generate cotton varieties with improved resistance to *Aspergillus flavus*. Varieties of cotton that are naturally resistant to *A. flavus* are not available, and conventional control measures are not effective in eliminating contamination. Our approach towards the development of cotton varieties with enhanced resistance to *A. flavus* focuses on the introduction of anti-fungal genes into cotton tissues using either biolistics, or *Agrobacterium tumefaciens* mediated transformation.

This strategy relies on three critical phases: identification, isolation and preparation of potential anti-fungal genes in a form that is amenable to the transformation of plant tissue, transformation of the plant tissue, and regeneration of transformed tissues for further propagation and analysis. We have identified a battery of potential anti-fungal genes that have been manipulated into genetic constructions to enable constitutive, tissue specific, developmental expression, or wound inducible regulation of gene expression. We have been working with a series of 11 constructs that encode potential anti-fungal proteins. Some of the proteins targeted for this study include: the basic bean chitinase, a chitinase/glucanase dual construct, osmotin, PGIP, and the Demeter anti-fungal peptide. We have used these constructs in both biolistic and *Agrobacterium* mediated transformation systems.

The second critical phase, introduction of new genetic material into plant cells, can be achieved either by *Agrobacterium* mediated transformation or bombardment with DNA coated particles (biolistics). *Agrobacterium* mediated transformation is a relatively efficient method for transforming many plants, but the biolistic approach has proven useful for plants or tissues that have either been recalcitrant to *Agrobacterium* mediated transformation, or difficult to regenerate following transformation. Our initial difficulty in regenerating cotton plants from *Agrobacterium* treated hypocotyl tissue led us to focus on a biolistic method for transforming cotton. The method that we used involved bombardment of meristematic tissues, selection of tissue for transformed cells, and growth of the meristematic tissue into mature plants. We experienced some success with this method, and identified some plants that had been transformed with an osmotin construct that tested positive with PCR and NPT analyses. However, when we tested progeny from these plants, we were unable to amplify osmotin specific DNA regions, although we could amplify native cotton DNA sequences. Thus we were not successful in obtaining "germ line" transformants which are essential for conventional breeding strategies that will be employed once a variety of cotton with enhanced resistance to *A. flavus* is developed.

Although *Agrobacterium* mediated transformation of cotton has been well documented as a reproducible method for obtaining stable, "germ line" transformants, this methodology requires successful regeneration of cotton from tissue - the third critical step in a genetic engineering

approach to develop cotton with new anti-fungal traits. In the past, in our hands, the time course for cotton regeneration has been prohibitively slow. However, we have returned to this method for generating transgenic cotton with enhanced resistance to *A. flavus*, and implemented some changes in our protocol based on recent publications. We also have developed optimized conditions for three different cotton tissues: cotyledon, hypocotyl, and meristem. Our experiments indicate that young cotyledon tissues, subjected to a modest vacuum in the presence of log phase *Agrobacterium* cultures can be induced to form callus under selection in 4-6 weeks. Kanamycin selection is very tight with cotyledon tissues--concentrations of 25ug per mL completely inhibit callus formation of non-transformed tissues. Hypocotyl sections from 6-12 day old seedlings, grown in the light, and treated with log phase *Agrobacterium* cells begin to form callus 2-3 weeks post infection. Kanamycin selection is effective with hypocotyl tissue, however, the break point is between 50 and 100 ug/mL. Both cotyledon and hypocotyl tissues must go through an embryogenic phase, "germinate", and then grow into mature plants. We have callus tissue on embryogenic medium at this point, but we have no figures on the time course for embryogenesis. Based on the most recent published data, we should obtain embryogenic cultures 8-10 weeks after callus formation. Meristematic tissues are treated with log phase bacteria, then plated onto selective media. They require no extensive regeneration protocols, but because meristematic tissue is somewhat resistant to kanamycin, our regimen involves frequent transfer to fresh selective media.

We are in the process of generating "germ line" transformants using an *Agrobacterium* mediated transformation system, followed by tissue culture to establish embryogenic cell lines, and subsequently develop transgenic plants. Because this is a lengthy process, we have also implemented an *Arabidopsis* based system to test our potential anti-flavus gene constructs *in planta*. The results obtained from these model plant studies will allow us to determine which constructs are most likely to be effective in transgenic cotton.

WHEAT SEED PR-4 AND TRYPSIN INHIBITOR HAVE SUBSTANTIAL *IN VITRO* ANTIFUNGAL ACTIVITIES AGAINST *ASPERGILLUS FLAVUS*

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Two low molecular weight proteins (13 and 8 kDa) were isolated and purified from the albumin fraction of wheat *(Triticum aestivum* var. San Pastore) seeds by chromatographic separation techniques and these proteins exhibit antifungal activities against *A. flavus.* The primary structure of the 13 kDa protein is homologous to pathogenesis-related (PR)-4 proteins, including about 95% identity with barley barwin and 70% identity with tobacco and tomato PR-4 proteins. This protein has been classified as a wheat PR-4 (wheatwin1). Evaluation of N-terminal sequence and trypsin inhibition data indicate that the 8 kDa protein is a wheat trypsin inhibitor (WTI) similar to the Bowman-Birk type of inhibitors. Wheatwin1 and WTI substantially inhibited *A. flavus* spore germination and germ tube elongation in a potato dextrose broth *in vitro* assay. The ED50 ($\mu g/\mu L$ of protein required for 50% inhibition relative to a control without protein) for hyphal growth and spore germination were 2.5 and 6.5 $\mu g/\mu L$, and 7.6 and 6.8 $\mu g/\mu L$ for wheatwinl and WTI, respectively. Using this assay, radish defensin, osmotin, or tobacco or grape chitinase were essentially ineffective as antifungal proteins against *A. flavus.* Current efforts are directed toward evaluating the additive/ synergistic *in vitro* antifungal activities of wheatwinl and WTI, and other antifungal proteins. Also, a wheat cDNA library will be obtained or constructed and the encoding cDNAs of these proteins will be isolated for expression of recombinant protein and genetic transformation.

GENE CONSTRUCTS ENCODING *ASPERGILLUS FLAVUS* GROWTH INHIBITORS FOR GENETIC ENGINEERING OF CROPS

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We have continued our research on the identification of proteins/peptides that demonstrate the ability *in vitro* to inhibit the growth of *Aspergillus flavus.* Additionally, we have constructed a variety of vectors for transfer and expression of genes encoding these candidate proteins/peptides in cotton. Finally, techniques for *Agrobacterium-mediated* transformation of cotton have been optimized which will enable these antifungal gene constructs to be more efficiently transferred into cotton and assayed for their ability to inhibit fungal invasion in plants.

A number of vectors have been constructed for the expression of the antifungal peptide D4E1. Of all antifungal proteins/peptides assayed to date, D4E1 has shown the greatest ability to inhibit the germination of *A. flavus* conidia. In addition, this peptide appears to be fairly resistant to degradation by proteases from *A. flavus* or cotton in comparison to the antimicrobial peptide cecropin which is rapidly degraded. We have placed a synthetic gene encoding D4E1 under the control of the CaMV and ubiquitin 3 constitutive promoters as well as a wound inducible potato protease inhibitor II (PIN II) promoter. In addition, D4E1 has been placed under the control of the cottonseed storage protein B-gene promoter that should limit expression of the gene to the seed only. These constructs have been transferred into Agrobacterium strains in preparation for transformation of cotton. Another gene product that has shown potential *in vitro* as an inhibitor of *A. flavus* growth is chloroperoxidase. It was demonstrated *in vitro* that a bacterial chloroperoxidase in conjunction with hydrogen peroxide (0.6 mM) reduced survival of germinated *A. flavus* conidia by 50%.

We have developed a system for the efficient transformation and regeneration of cotton with the above mentioned antifungal constructs. Using *Agrobacterium*-mediated transformation technology, we have successfully regenerated mature plants with a stably integrated copy of the GUS reporter gene which was expressed at high levels in embryogenic callus as well as young leaf tissues.

REDUCED *A. FLAVUS* SPORULATION ON TRANSGENIC WALNUT EMBRYOS

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Genetic engineering offers the prospect of reducing aflatoxin contamination by inserting and expressing foreign genes directly in elite crop cultivars. An important first step is to identify genes which can provide a useful degree of protection against *Aspergillus flavus* itself, or against its insect vectors. Walnut provides a good model system in which to test potential anti-fungal genes because transformation of this species is relatively routine. In addition, the initial regenerant in the walnut transformation system, the transgenic somatic embryo, multiplies readily and can be used to test the efficacy of the inserted genes against *A. flavus*. Somatic embryo lines containing nettle and barley lectin genes, and three lines containing the Demeter Peptidyl MIM ™ D5D significantly reduced sporulation of *A. flavus* in our bioassay, as compared to the untransformed control. Embryo lines containing the chitin-binding gene hevein, two additional D5D lines, and one line containing snowdrop lectin reduced sporulation somewhat, but the reduction was not statistically significant. Six embryo lines with the systemic acquired resistance gene SAR 8.2 and three with snowdrop lectin did not perform better than the control embryos. Scanning electron microscopy revealed that fungal hyphae growing on transgenic walnut embryos containing hevein, or the nettle and barley lectins, had a tendency to appear lumpy and deformed as compared to hyphae growing on untransformed embryos.

PROMOTER FUNCTION AND PEST RESISTANCE IN TRANSGENIC PEANUT

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As a resistance strategy, transgenes can be expressed constitutively or can be designed to respond to developmental, chemical, or environmental cues for expression that is controlled to provide optimal exposure of the target pest to the gene product. This approach should reduce the constant exposure of a pest to toxic gene products and might reduce the probability for the pest to develop resistance. The widely used cauliflower mosaic virus 35S (CaMV 35S) promoter not only is less effective in some species, but also expresses any gene under its control in a nominally constitutive pattern. One promoter we have focused the present work on is derived from a soybean vegetative storage protein gene. This gene is developmentally regulated in the sense that its product accumulates to high levels in actively growing tissues such as the soybean pod wall and the hypocotyl of germinating seeds. The *vspB* gene expression is modulated by carbohydrates, wounding, methyl jasmonate, phosphate, auxin, and water deficit. By targeting expression of a resistance gene such as Bt to its most needed site of action, we reduce the diversion of plant resources to transgene products and reduce the potential for negative effects on insect resistance management while maintaining effective control of the target pest. With this future resistance management strategy in mind, we initially tested the activity in peanut of the *vspB* promoter when fused with the *uidA* coding region. Over 200 plants from 39 cell lines PCR-positive for the *vsp-uidA* gene were regenerated. All cell lines subjected to the regeneration protocol produced plants. Approximately three months were needed for plant regeneration from embryogenic culture and about four additional months were required for plant development (growth, flowering, pegging and seed pod maturation) in the greenhouse. Over 84% of the cell lines flowered; however, only 50% of the cell lines produced pods. Approximately 1000 seeds were recovered from 79 fertile R_0 plants. Seventy-five of the fertile plants were tested by PCR for the *vsp-uidA* fragment and 73 showed good amplification. R_1, plants were grown in the greenhouse over the summer. Several thousand R_2 seeds have now been harvested and will provide material for more detailed gene expression and genetic analysis in the 1997 field season.

GUS activity in transgenic tissues and organs was modulated by jasmonic acid, phosphate deficit and mannose. Although the GUS activities were different among different cell lines, the average GUS activities increased approximately 3- to 5-fold when transgenic tissues from seven lines were exposed to 50 uM jasmonic acid, either in the presence or absence of 7% mannose. The expression of GUS driven by the *vspB* promoter was developmentally regulated with low activity in roots, moderate activity in leaves, and the highest activity in stems of three primary transgenic plants. As observed with expression in cell lines, expression varied greatly among transgenic plants of independent origin even though stems from the same developmental position on each plant were sampled.

From a single progeny plant recovered from our first transgenic Bt plants, several R_2 seed have been collected. Several hundred R_1 seed also have been collected from a more recent experiment designed to recover fertile peanut plants containing the Bt toxin gene. A second generation will be planted in the greenhouse this winter, and R_2 seed will be collected for a replicated field test next summer that will attempt to combine lesser cornstalk borer infestation and *Aspergillus* invasion.

TRANSFORMATION OF PEANUT WITH GENES ENCODING ANTIFUNGAL PEPTIDES

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The primary goal of this project is to decrease the probability of aflatoxin contamination of peanuts by transforming with genes encoding antifungal peptides. Peptides with selective activity against fungi could be expressed in transformed plants to inhibit growth and reproduction of invading fungi, reducing the severity of mycotoxin contamination and the probability of its occurrence. This is a report of progress made during 1995-1996 toward development of such a system, incorporating either the proprietary Membrane Interactive Molecule® (MIM) D5C (Demeter Biotechnologies, Ltd.), or the naturally-occurring protein, osmotin, derived from tobacco.

D5C is one of a class of synthetic peptides modeled after the structure of cecropins. The peptide, which forms an amphipathic α-helix, is thought to insert into the cell membrane to form pores which result in osmotic shock. D5C inhibits the growth of both *Aspergillus flavus* and *Cercospora arachidicola, in vitro*. Osmotin is a larger peptide with demonstrated activity against a broad range of fungi *in vitro*. It does not exhibit strong activity against *Aspergillus* when used alone, but exhibits strong synergistic activity when combined with chitinase.

Several key questions must be answered if such peptides are to be used effectively. D5C showed strong activity against *A. flavus in vitro*. However, this observation left unanswered questions about the phytotoxicity of the constitutively expressed peptides, which might complicate recovery of transgenic plants. Toxic effects of the peptides could eliminate transgenic cells prior to plant regeneration, or could affect fertility of transformed plants. Further, it was not known whether observations *in vitro* would accurately predict efficacy against fungal growth *in vivo*.

A tobacco model system was used in parallel with peanut to study transformation with genes encoding D5C. Tobacco, is easier and faster to transform than peanut, and transgenics can be obtained in relatively large numbers. Tobacco also has numerous fungal pathogens, facilitating test of antifungal activity. Two burley tobacco lines were transformed with D5C. Transformation was verified by Southern blots probed with the D5C, and Northern blot (mRNA) analysis. Preliminary Western blots are consistent with production of D5C. Detached leaves were inoculated with *Rhizoctonia* or *Phytophthora*. Symptom development was delayed in transgenic leaves, and symptoms did not ever become as severe as those observed on controls.

D5C was also transferred into peanut cv. NC 7. Molecular characterization of primary transgenics and their self-progeny are consistent with integration of the transgene, although transcription and peptide synthesis have not yet been demonstrated. An efficacy trial carried

out with clonally propagated R1 progeny of resistant primary transformants showed apparent reduction of symptoms in some lines inoculated with the test fungus, *Cercospora arachidicola.*

Agrobacterium based DNA transfer systems have also been investigated and optimized because of the need for reliable co-integration of the defensive peptide and a selectable marker gene, and the systems inherent efficiency. Transformation protocols based on *Agrobacterium*-mediated gene transfer to peanut cv. NM Valencia A have been radically improved by the use of feeder cultures derived from tobacco cv. Burley 21. Improvements to published protocols greatly enhanced system throughout and transformation efficiency. The improved protocol was used to introduce a gene encoding osmotin into NM Valencia A. PCR analysis of transformed lines was consistent with genomic integration of the osmotin gene into primary transformants, and subsequent transmission to progeny. The first test of fungal resistance in these transformants is currently underway in a replicated trial inoculated with *Cercospora arachidicola.* Lines carrying the osmotin gene were also entered into a backcross program, with VAC 92R as the recurrent parent. BC1 seed has been produced. Progeny will be screened for presence of the gene and positive individuals will be used to produce the BC2 generation in the coming year.

Although a great deal of additional work is required to demonstrate efficacy of antifungal peptides in peanut, these results suggest the utility of this approach as part of a comprehensive aflatoxin elimination strategy.

POSTER PRESENTATIONS

ENGINEERING AND CLONING OF SEED LIPOXYGENASES IN PEANUT

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Lipoxygenase, a stress response enzyme in plants, adds 0_2 to the polyunsaturated C-18 fatty acids, linoleic and linolenic acid, to produce hydroperoxy fatty acids which have been implicated in plant defense against fungi. We have previously shown that the linoleic acid product of soybean lipoxygenasel, 13(S)hydroperoxy linoleic acid (13S-HPODE), decreased aflatoxin (AF) and sterigmatocystin (ST) production and AF/ST gene transcription in *Aspergillus spp.* grown in vitro. To determine if this compound acts to inhibit AF production in vivo, we are transforming two cultivars of peanut (Georgia Runner and Florunner) with the soybean lipoxygenasel gene, LOX1. Embryogenic calli were transformed with a vector carrying both LOX1 and HPT, a gene encoding resistance to hygromycin, using microprojectile bombardment methods. Tissue culture selection for resistance to hygromycin has resulted in the recovery of 26 and 50 putative transformants of Georgia Runner and Florunner, respectively. We are in the process of verifying if the putative peanut transformants incorporated LOX1 into their genomes.

In addition, we are isolating peanut lipoxygenase genes from a peanut cDNA lambda phage library made from 20 d old seed. We have isolated one clone, Cl-lC, from the library which strongly hybridizes to soybean LOX1. We will clone and sequence the insert to verify if it is a lipoxygenase homologue.

INTRODUCTION

Aflatoxin (AF) contamination of susceptible crops (e.g. peanut, corn, cottonseed, almonds, to name a few) poses a serious health hazard to consumers and an economic loss to farmers. To alleviate this problem, rational control methods that are geared towards reduction and/or elimination of AF in the crops of interest must be developed. Our approach towards this goal is through the identification, characterization and genetic manipulation of plant genes which produce products that inhibit or suppress AF production. For this project, we are focusing on the plant lipoxygenase pathway as its products have been implicated as contributing to soybean and cotton resistance to infection by *Aspergillus flavus* (Doehlert et al., 1993; Zeringue, 1996). Our in vitro results show that the main product of soybean lipoxygenasel, 13(S)-hydroperoxy linoleic acid, suppresses the expression of toxin genes and toxin production in both AF and sterigmatocystin (ST) producing *Aspergillus spp.* (Burow et al., submitted). We are pursuing this finding in vivo by asking if (i) an increase in the level of 13(S)-hydroperoxy linoleic acid (13S-HPODE) in peanut seed will suppress or inhibit AF production in peanut, and (ii) if naturally occurring peanut lipoxygenases play a role in *Aspergillus* colonization of peanut seed. The objectives of this present study are:

1. To transform peanut with soybean lipoxygenasel (e.g., LOX1) and assess the effects of LOX1 expression in transformed peanut on susceptibility or resistance to *Aspergillus* colonization and AF production.

2. To identify and characterize lipoxygenase gene(s) in peanut and determine the role of these genes in the peanut-*Aspergillus* interaction.

DISCUSSION

We have previously demonstrated that the addition of 1-100 μM 13-(S)HPODE could alter the expression of AF/ST genes and toxin production in *Aspergillus* spp. grown in liquid shake culture (Burow et al., submitted). Specifically, 13-(S)HPODE initially represses *ver-1* and *stcU* transcript and subsequent AF/ST production in *A. parasiticus and A. nidulans*, respectively. We have also analyzed the effects of 1000 μM 13-(S)HPODE on AF production in *A. parasiticus* and found that this amount of 13-(S)HPODE completely eliminated AF production in *A. parasiticus* ATCC98016 even after 10 days growth of the fungus (Burow et al., submitted).

Based on these results we are investigating whether 13-(S)HPODE could also function in planta to inhibit AF biosynthesis and thus confer resistance to susceptible crops. Peanut embryogenic calli were transformed with the plasmids pGB4 and pGB5 by microprojectile bombardment. Each plasmid contained both the soybean LOX1 and *E. coli* hygromycin B resistance gene. Putative transformed calli were selected in liquid media supplemented with 20 mg/L hygromycin. A total of 570 peanut embryogenic calli were bombarded and after selection in hygromycin containing liquid media (20 mg/L hygromycin), 76 putative transformants were recovered and grown for regeneration. This represents a 13.3% recovery of putative transgenic cell line, which was similar to the value reported earlier for this transformation method of peanut (Ozias-Akins et al., 1993). Current studies are underway to verify the integration of LOX1 into the possible transformants by PCR and southern blot methods. Positive transformants will be further tested by Northern analysis and lipoxygenase activity assays.

We have also initiated studies on the isolation and characterization of lipoxygenase gene(s) in peanut (earlier studies have suggested that there are at least two peanut seed lipoxygenases (Pattee et al., 1974; Sanders et al., 1975). A λgt11 cDNA library from 20 day old peanut seeds was screened by hybridization to soybean LOX1. Two rounds of screening resulted in identifying a putative clone, designated as clonelC, which strongly hybridizes to LOX1. Due to its size and nucleic acid hybridization properties we believe that we have identified a strong candidate for a peanut seed homologue to soybean LOX1. We are presently sequencing this clone to verify its identity.

SUMMARY AND FUTURE DIRECTIONS

We are continuing to study in detail the role of lipoxygenase and its products in controlling AF production in the plant by transforming peanut cultivars with soybean LOX1. We have recovered putative transgenic peanut plants using microprojectile bombardment. In the next year, we will assess whether these putative transgenic plants express LOX1 and, if so, determine if they have enhanced resistance to *Aspergillus* colonization and AF contamination. To better understand the interaction between lipoxygenase expression and *Aspergillus* infections, we have started cloning lipoxygenase gene(s) from peanut seed. Areas we will pursue in the following year:

1. Molecular analysis of putative transgenic peanuts to verify if they are transformed with LOX1.

2. Construction of improved soybean LOX1 transformation vectors for peanut transformation.

3. Evaluation of LOX1 transgenic peanut seed to *Aspergillus* infection and AF production.

4. Isolation and characterization of peanut lipoxygenase genes and assessment of their role in fungal/plant interactions.

ACKNOWLEDGMENTS

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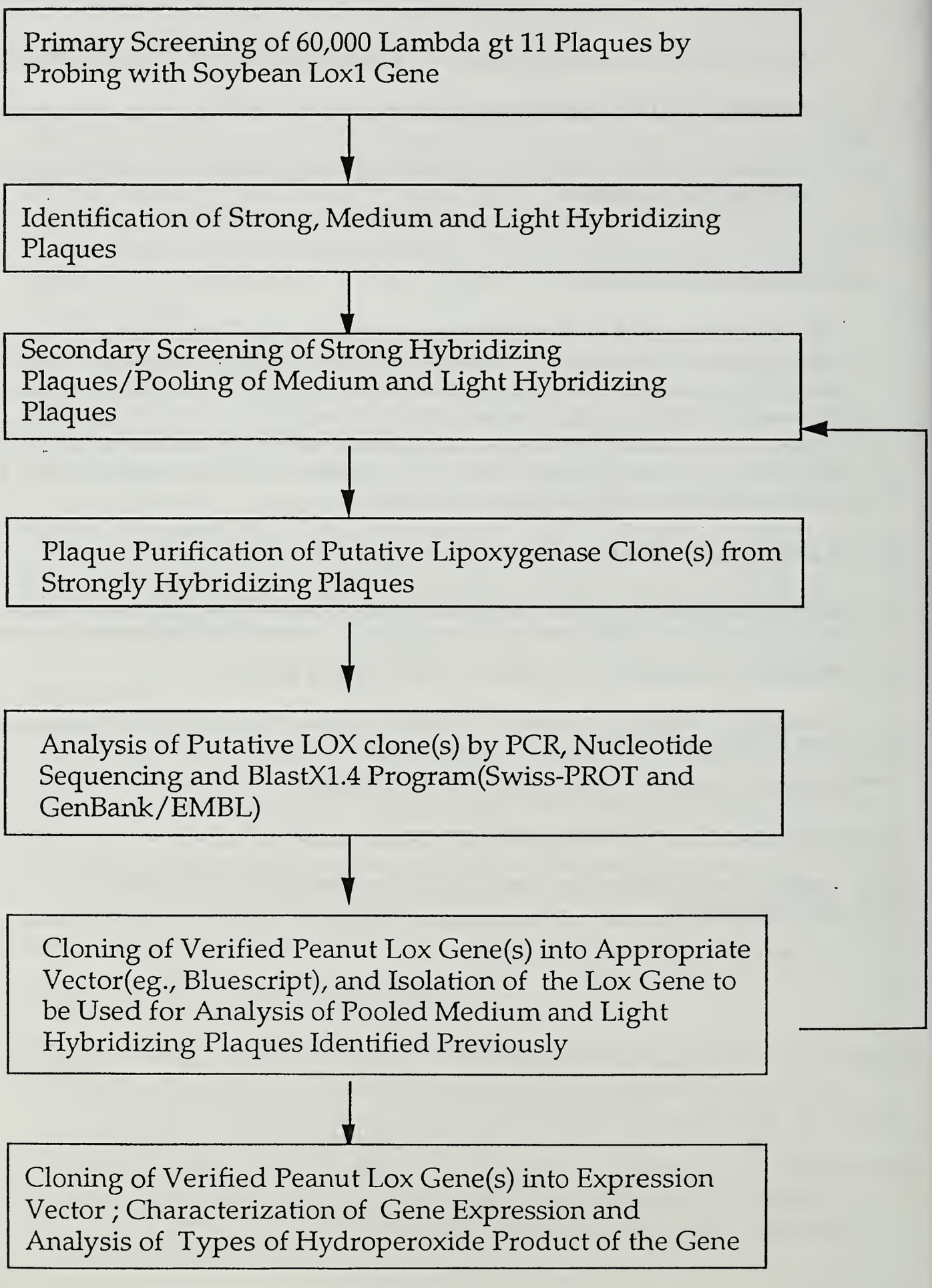
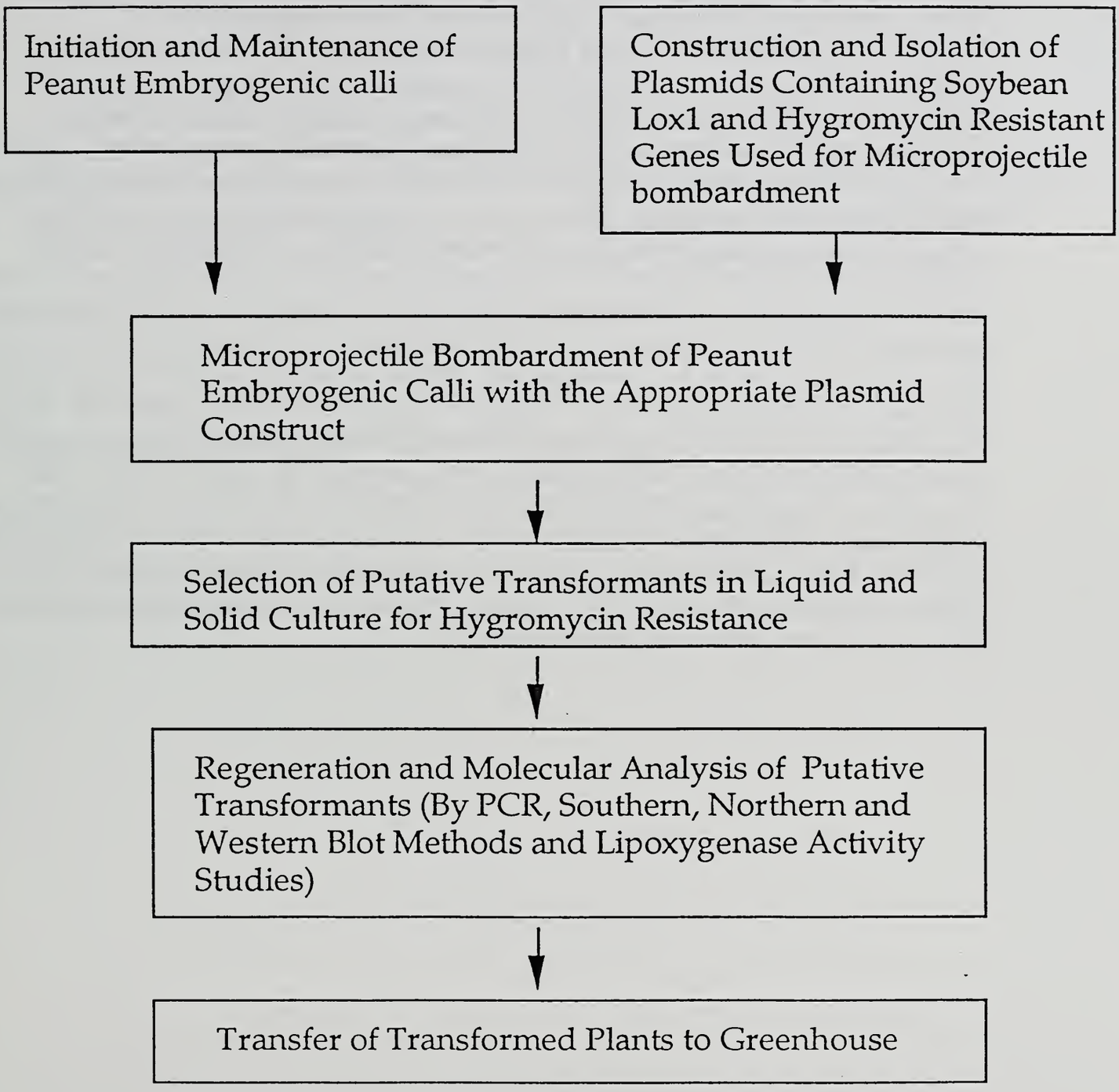
FLOWCHART OF cDNA LIBRARY SCREENING FOR CLONING PEANUT SEED LIPOXYGENASE GENE(S)



FLOWCHART OF PEANUT TRANSFORMATION WITH SOYBEAN LOX1 AND REGENERATION OF TRANSGENIC PLANTS



ANTIFUNGAL PROPERTIES OF THE NATURAL PEPTIDES CECROPIN B AND DERMASEPTIN

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Peptide genes are being sought which encode potent antifungal peptides for genetic engineering of plants resistant to *Aspergillus flavus/parasiticus*. *In vitro* antifungal properties of cecropin B (CB) and dermaseptin (DERM), potent antibacterial peptides from the giant silk moth and frog skin, respectively, were explored. Nongerminated and germinated (8 hr) conidia from *Aspergillus flavus* (AF), *A. fumigatus* (AFUM), *A. niger* (AN), *Fusarium moniliforme* (FM) and *F. oxysporum* (FO) were incubated (30 min, 30°C) separately with the peptides. Aliquots were inoculated onto PDA plates, incubated (24 hrs, 30°C) and colonies enumerated. Neither peptide reduced conidial viabilities of nongerminated *Aspergillus* spp. CB produced LD_{50} values for germinated AF, AFUM, and AN conidia of 3.0, 0.5, and 2.0 μM, respectively, while DERM gave LD_{50} values of 4.0, 0.05, and 2.0 μM, respectively. CB gave LD_{50} values of 0.2 μM for nongerminated FM and FO conidia, while DERM only slightly reduced viabilities of both *Fusarium* species. LD_{50} levels for CB were 0.2 and 0.1 μM, respectively, for germinated FM and FO conidia. DERM was less effective, giving LD_{50} values for germinated FM and FO conidia of 0.3 and 0.8 μM, respectively. Physicochemical studies indicated CB and DERM bound to ergosterol, a constituent of fungal cell walls. Neither peptide complexed with chitin or β-1,3-glucan. Results show that antifungal properties of these peptides were genus and species dependent.

POTENTIAL OF CHLOROPEROXIDASE AND MYELOPEROXIDASE TO PROTECT CROPS FROM AFLATOXIGENIC FUNGI

T.J. Jacks, A.J. De Lucca, P.J. Cotty and T.E. Cleveland, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

We evaluated the potential of chloroperoxidase (CPO) and myeloperoxidase (MPO) to improve plant defense against aflatoxigenic fungi. The natural defense of plants, hydrogen peroxide (H_2O_2), was found moderately lethal to *Aspergillus flavus*. CPO and MPO increased H_2O_2-lethality by up to 90-fold, equivalent to hypochlorous acid [(HOCl), the active biocide in Chlorox^R]. The magnitude of gain indicates that acquisition of CPO or MPO such as by genetic engineering would greatly boost defense capacity against fungal infection.

CHARACTERIZING THE NATURAL RESISTANCE OF PEANUT TO *ASPERGILLUS PARASITICUS* AND AFLATOXIN

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We hypothesize that there are specific factors in resistant lines of peanut that repress the invasion of *Aspergillus parasiticus* into the peanut pod and the synthesis of aflatoxin in the pod. The timing of induction and shutdown of expression of these resistance factors may be responsible for the resistance breakdown observed under certain field conditions (most notably drought and high temperatures). Direct invasion of the peanut kernels from the soil is the most significant source of infection in peanut, according to Cole et al. (1986: Appl. Environ. Microb. 52: 1128-1131). We seek to identify the natural resistance mechanisms present in the developing peanut pod that are important to limiting fungal growth and aflatoxin production initially by detecting the timing and location of known resistance genes in infected pods. Several peanut lines are now available that appear to have differing resistance mechanisms as observed in the field. It is likely that multiple resistance mechanisms are involved in these peanut lines. Peanut lines were inoculated with a genetically engineered strain of *A. parasiticus* (G5) and strain NRRL 6111, a strain commonly used in field studies. Our results showed that both strains are pathogenic on drought stressed plants. The genetically engineered strain is used to infect resistant and susceptible lines to detect the timing and location of aflatoxin production in pods of different ages and grown under different conditions. These infected kernels are then used in studies to detect expression of genes involved in resistance. We have constructed DNA vectors containing portions of the resveratrol synthase (a putative plant resistance gene) and the vicillin (a seed storage protein to be used as a control) genes. *In situ* hybridization will be used to determine the areas where these genes are expressed and in kernels of different ages. As yet uncharacterized resistance mechanisms affecting aflatoxin production and infection can be detected similarly by use of genetically engineered strains of *A. parasiticus* and by observation of infection at the cellular level. These new mechanisms can then be characterized. Once resistance mechanisms have been identified that are effective, their expression may be optimized through genetic engineering so that the resistance is protective over a longer period of time, or simply the correct period of time.

CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Integration of Crop Management Techniques with Other Promising Strategies to Control Aflatoxin

PANEL MEMBERS: T. J. Michailides (Chair), M. A. Doster, T. Isakeit, T. Pearson, T. Schatzki, P. Dowd and G. Windham

SUMMARY OF PRESENTATIONS: The speakers presented the progress of their research since the last Aflatoxin Workshop in Atlanta:

Doster reported on studies in Michailides' laboratory that demonstrated delaying pistachio harvests did not increase shell split of normal pistachios, but increased incidence of early splits and shell discoloration. In 1995, the fig irrigation experiments showed that increasing irrigation decreased both aflatoxin levels and incidence of BGY fluorescence, results consistent with those of the 1994 study. In inoculation experiments, wounding substantially increased aflatoxin in green and yellow figs, but not in brown figs, which might explain why insect damage to mature figs does not result in increased aflatoxin contamination.

Isakeit reported on the relationship of insect injury of cotton bolls and aflatoxin contamination and showed that damaged tight lint had more BGY fluorescence and aflatoxin than fluffy damaged lint. In addition, aflatoxin increased significantly during the post-rain period. His survey showed that in the absence of weathering, insect injury was the main factor leading to aflatoxin contamination.

Schatzki reported on the distributions of pistachios with aflatoxin and their importance in aflatoxin analysis and interpretation of aflatoxin distributions. The distributions matched the shape and position of those derived from early split nuts computed from previous studies. In almonds, almost all the aflatoxin comes from ground and chopped manufacturing stock. He also reported on the use of X-ray technology for testing insect-infested vs. non-infested pistachio nuts.

Pearson has improved color sorting machines so that they can detect and sort out pistachio nuts with the characteristic early split suture stain. Patents for this sorting machine have been submitted.

Dowd reported on the potential for corn expressing Bt crystal protein to indirectly control mycotoxigenic fungi through caterpillar control. Expression of the Bt protein is high in green tissue and pollen, and much lower in other tissues. Despite the limited expression, the commercial Bt hybrid tested has the potential to substantially reduce mold incidence compared to the non-Bt hybrids.

Windham reported on the effects of the southwestern corn borer (SWCB) on aflatoxin contamination and *Aspergillus flavus* kernel infection in corn in field experiments. In 1995, aflatoxin levels in all hybrids tested were higher when treated with both SWCB and *A. flavus*. In 1996, however, SWCB had no apparent effect on aflatoxin contamination. Thus, although the

damage caused to kernels by SWCB can lead to substantially increased aflatoxin levels, results also depend on method of inoculation and site of placement of the pests on plants.

SUMMARY OF PANEL DISCUSSION: Early discussion began with questions about data which Tom Pearson had presented which showed a relatively high percent of good pistachios being rejected by a color sorter that detects flawed nuts based on stain. The economics of a system that rejected so many good nuts was questioned. Pearson responded by saying that re-sorting is necessary to retrieve initially rejected good nuts, a practice that is done even with current sorting procedures. Schatzki also reinforced this statement that re-sorting is necessary which also cuts down the cost of hand sorting. Hua asked whether there was a similar situation (as with early splits) in peanuts. Cutchins stated that the peanut industry does sort, but it is necessary for the industry to use as much of the product as possible. Tozun asked Pearson how much of the rejected product was used, and Pearson responded that more than 90% of the rejected product could be used if it could be recovered.

Jones asked Doster whether different water potentials on the surface of the soil influenced aflatoxin levels in figs. Doster explained that this is a complex situation to assess because fig fruits are at different stages of maturity when they fall onto the ground, and the soil moisture varies from location to location under each tree.

Widstrom questioned how irrigation treatments affected competition of *Aspergillus* fungi by other microorganisms. Doster responded that, although he had recorded other microorganisms, the analyses of the data had not been completed, therefore, it was difficult for him to say anything more on the effects the other microorganisms played in the irrigation experiment. Cotty asked what was the highest level of aflatoxin detected in figs. Michailides mentioned that they had isolated individual figs with up to 77,000 ppb. However, as Schatzki mentioned, the levels of aflatoxin in a sample will depend both on the distribution of aflatoxin within the sample and on the sample size. For instance, the DFA uses large samples of figs (up to 35 lbs) for aflatoxin analysis.

Wicklow asked Isakeit whether there was any varietal resistance of cotton to aflatoxin contamination, or if there was a breeding program to develop aflatoxin resistant varieties. Isakeit responded that aflatoxin is not the main goal in breeding programs for cotton.

In response to Dowd's question regarding the relative value of the cottonseed in comparison with the lint, Isakeit responded that cotton is grown primarily for the lint and the seed is a by-product. However, Wakelyn added that seed represents 10 to 15% of the value of the crop, and since aflatoxin is not oil soluble, cottonseed oil is free of aflatoxin. Isakeit added that some of the cotton gin trash is used for animal feed and it may contain a lot of aflatoxin.

Jones added that transgenic Bt cotton was first planted in 1995. With the exception of some pockets in cotton growing areas experiencing insect damage, it worked well, and he expected its use to increase in the next year. Similarly, Bt transgenic soybeans have been planted on about 2 million acres grown in USA.

Michailides asked if there had been any reports on resistance to Bt. Jones said that DuPont has anticipated this. This situation could be avoided by using genes for Bt proteins showing different

characteristics (mode of action) thereby circumventing resistance buildup in insect populations to a particular Bt protein.

Betschart asked whether the choice of marker genes in transgenic plants could be a problem if the pests also develop resistance to the marker genes, and whether this could be a human consumption issue. Sacher said that there was an example from the UK only, where the DNA that was transformed into corn has not been a consumer issue.

Finally, Dowd said that the European Corn Borer which developed resistance to Diapel showed no cross resistance to other pesticides.

Michailides concluded that similarly to the other panels focusing on other control strategies, significant progress has been made towards identifying sources of and developing field control methods for aflatoxin in tree nuts, figs, cottonseed, and corn. Progress has also been made in developing methodology and systems for separating the aflatoxin-contaminated fraction of the commodity from the non-contaminated crop.

PLATFORM PRESENTATIONS

CULTURAL PRACTICES THAT REDUCE AFLATOXIN CONTAMINATION AND CHARACTERISTICS FOR REMOVAL OF CONTAMINATED FIGS AND PISTACHIO NUTS

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Pistachios. Studies were initiated to determine the effect of harvest date on fungal and aflatoxin contamination of pistachio nuts. Nut samples were harvested from 30 trees in a commercial orchard on 3, 13, and 23 September. The last harvest had a higher percentage of early splits (abnormal nuts with split hulls, and the main source of aflatoxin contamination) and more discoloration of the shells than the first harvest, while the percentage of shell split of normal nuts was approximately the same for all harvests (78, 75, and 75% for the first, second, and last harvest, respectively). The evaluation of the nut samples for fungal contamination is in progress. In another study, hydrogen cyanamide (which induces pistachio trees to break dormancy early) increased the percentage of early splits at harvest (9.3 and 2.3% for the hydrogen cyanamide treatment and for the control, respectively).

Figs. Irrigation treatments ranging from 75 to 225% of normal water requirements were applied in a Calimyrna fig orchard. Similar to the results of the previous year, the incidence of figs colonized by *Aspergillus* Section *Flavi* and the incidence of figs with BGY fluorescence decreased with increasing amounts of applied water in the summer. Although increased irrigation did not have an obvious effect on the levels of propagules of *Aspergillus* Section *Flavi* on leaves, increased irrigation did result in larger ostioles of the figs and in higher incidences of these fungi colonizing noncaprified figs on the ground. In another study, figs left on the ground after harvest and examined on 9 November were found to have an unusually high incidence (0.7% of the figs) of colonies of *Aspergillus* Section *Flavi*.

Further studies on the use of bright greenish yellow (BGY) fluorescence for removing contaminated figs were performed. Calimyrna figs in a research orchard were inoculated with six isolates in *Aspergillus* Section *Flavi*. For these isolates, between 91 and 97% of the infected figs showed BGY fluorescence, even though three isolates were originally from figs with no BGY fluorescence. Only 82% of the figs with colonies of *Aspergillus* Section *Flavi* smaller than 11 mm diameter were BGY fluorescent, whereas all figs with colonies greater than 20 mm were BGY fluorescent. Also, figs with large internal colonies (> 20 mm diameter) were more likely to have externally visible BGY fluorescence than figs with colonies less than 11 mm diameter. In another study using Conadria figs, infected figs that had not started drying down were more likely to be BGY fluorescent than infected figs that were at a lower moisture content.

Calimyrna figs were artificially wounded and inoculated with *A. flavus* in a research orchard in August 1996 in an experiment similar to one performed in 1995. Wounding of figs resulted in more infections by *A. flavus* for green figs with the eye open and for yellow figs. For very immature figs (green with eye closed) and very mature figs (brown), however, wounding did not result in a significant increase in infections. Aflatoxin analysis of the figs from the 1995

experiment showed that wounding substantially increased aflatoxin production for green figs (0.00 and 0.88 mg aflatoxins per fig for nonwounded and wounded, respectively) and for yellow figs (0.26 and 1.209 mg) but not for the mature brown figs (0.44 and 0.23 mg). These results suggest that damage to mature brown figs does not favor aflatoxin production, which might explain why insect damage to the mature fig does not result in increased aflatoxin contamination in figs.

THE RELATIONSHIP OF INSECT INJURY TO AFLATOXIN CONTAMINATION OF COTTONSEED IN SOUTH TEXAS

T. Isakeit, Texas A&M University Research and Extension Center, Weslaco, TX.

The main objective of this study was to determine whether aflatoxin contamination of cottonseed was associated with insect injury to bolls. The contribution of weathering (exposure to rain over four weeks) of mature cotton in the field to aflatoxin contamination was also monitored. Samples were taken from fields and modules located in four counties in the Lower Rio Grande Valley and the Coastal Bend. Seed from damaged locks were analyzed separately from non-damaged locks. In the case of damaged locks, seed associated with bright green yellow fluorescent (BGYF) lint, indicating *Aspergillus flavus* infection prior to boll maturity, was analyzed separately from seed originating from other, non-BGYF, insect-damaged locks.

There was no aflatoxin contamination of cottonseed originating from non-damaged bolls or non-damaged locks from insect-damaged bolls in samples from thirteen fields that had not been weathered. In contrast, 61% of seed from insect-damaged locks had high (i.e., >20 ppb) aflatoxin concentrations, with an average concentration of 649 ppb. Cottonseed samples from modules originating from fields that had not been weathered showed a similar association of high aflatoxin concentrations with insect injury. Locks showing evidence of insect injury were present in 70% of 30 module samples. Of these seed samples, 53% associated with BGYF lint and 37% associated with insect-damaged, non-BGYF lint had high levels of aflatoxin. The aflatoxin concentration ranges in these samples were 35-2500 ppb and 27-2200 ppb, respectively. In contrast, only sample consisting of non-damaged locks had a high aflatoxin content (160 ppb). The primary insect causing boll damage in the 1996 season was the boll weevil. The cotton bollworm was present to a lesser extent.

Exposure of mature cotton in the field to rain showers over a period of four weeks resulted in high levels of aflatoxin contamination of all types of samples. While one sample (i.e., 3%) of seed with non-damaged lint from modules made before this rainy period had high levels of aflatoxin, 82% of such samples from modules made after the rainy period had high levels of aflatoxin. The mean aflatoxin concentration of these samples was 760 ppb. The aflatoxin contamination of cottonseed from non-damaged locks collected from two fields following severe weathering was 280 ppb and 150 ppb, while the contamination of corresponding insect-damaged samples was 930 ppb and 1400 ppb, respectively.

This survey shows that in the absence of weathering, insect injury is the main factor leading to aflatoxin contamination. The presence of BGYF lint is highly correlated with contaminated cottonseed, but seed associated with non-BGYF insect-damaged lint was also highly contaminated. However, severe weathering in the form of heavy rain over a period of several weeks can result in high levels of aflatoxin contamination.

MACHINE VISION SYSTEM FOR REMOVAL OF AFLATOXIN CONTAMINATED PISTACHIO NUTS

Tom Pearson, USDA, ARS, Western Regional Research Center, Albany, CA.

Background:

The pistachio processing industry in California currently utilizes machines called "automatic color sorters" to sort for stained pistachio nuts. However, there are serious limitations with using the color sorters to sort pistachio nuts with the characteristic early split suture stain. Color sorters measure the "average color" of the entire nut surface. If the average color exceeds a certain threshold of brownness, then the nut will be ejected from the process stream. One problem with this sorting criteria is that if the size of a stain is relatively small, as many early split suture stains are, then it will not contribute very much to the average color of the shell surface. This type of error leads to early split nuts passing into a higher quality process stream which may reach the consumer. Another problem with the color sorters is that the shell split width opening on pistachio nuts can be quite variable which exposes, to different degrees, the dark brown kernel inside the shell. The kernel color can significantly alter the average color of the pistachio surface and cause completely unstained nuts with large shell split opening to be erroneously removed by the color sorters. This type of error causes high quality product to be mixed with a very low value stained product. The machine vision system developed overcomes both of these problems by counting the number of discolored regions on the nut surface. All nuts will have two discolored due to kernel exposure through the shell split on each side of the nut. However, this criteria is independent of the size of the split opening. Furthermore, the size of the stain is independent of this criteria as well. Thus, the characteristic early split suture stain can reliably be detected with this sorting technique without accumulating several false positives comprising unstained nuts with large split openings.

Progress since Fall 1995:

The machine vision system was tested at a California pistachio processor on their color sort reject nuts. This processor operates their color sorters in such a way that they remove most of the stained nuts, including the early split suture stained nuts. However, this reject stream also contains many nuts which are relatively clean but have large shell split openings as a result of the vigorous stain sorting of the color sorters. Approximately 10% of this processors incoming nuts are rejected by their color sorters. One thousand seven hundred pounds of pistachio nuts were sorted with the machine vision system, 47% were classified as stained, while 53% were recovered as unstained. Aflatoxin analysis showed that the nut classified by the machine vision system as stained contained a mean aflatoxin level of 19.1 +/- 5.2 based on 160 samples. In contrast, nuts classified by the machine vision system as clean had a mean aflatoxin content of 0.04 +/- 0.01 based on 100 samples. For this analysis, one sample comprised 500 nuts or about 1.5 pounds.

ELIMINATION OF AFLATOXIN IN TREE NUTS THROUGH POST-HARVEST SORTING

T. Schatzki, CPU, USDA, ARS, Western Regional Research Center, Albany, CA

Measurements on wet pistachios, mentioned last year, have been repeated. Whereas the samples last year were derived from taking nuts out of the processing stream during harvest (after the floatation bath), in the present experiment 1000 lb. bins were selected from the orchard. Each bin represents about 6 trees, without mixing. While last year a rapid rise of aflatoxin was observed after 4 days storage if the water content exceeded 10%, virtually no aflatoxin was observed in the present experiments. Presumably, the 6 trees in question were aflatoxin free, since no opportunity for cross contamination existed.

Aflatoxin results in small pistachios (scalpers) have been reanalyzed. Scalpers from one processor have been found to have an average aflatoxin of 8 ppb, not inconsistent with 24 ppb, previously reported by that processor [Schatzki, T.F. and Pan, J.L. Distribution of aflatoxin in pistachios. 3 Distribution in process streams. J Agric. Food Chem. 44(4):1076-1084, 2468]. Size dependence, if any, is very small. This average is well below that of another processor previously reported by us (Schatzki, loc. cit.), which came to 170 ppb. This points to a difference in the technique of scalping between the processors.

The distribution of aflatoxin in a single lot of pistachios (a re-sort of color sorter rejects, with average aflatoxin content of 19 ppb) has been computed based on the results on 160 500-nut samples. This distribution matches in shape and position almost exactly that derived from early split nuts, computed [Schatzki, T.F., J. Agric. Food Chem. 43:1566-1569(1995)] from Sommer's data [Sommer et al. Phytopath.76:692-694 (1986)] and closely matches that computed from mixed lots (Schatzki, loc. cit.). Among these samples, none exceeded 270 ppb, a somewhat lower value than would be predicted if nuts with up to 1,000,000 ppb aflatoxin were present. Sample measurements will be continued until 400 samples are done.

The distribution of aflatoxin among almond process streams for crop year 1993 has been estimated on the basis of laboratory data covering 78% of total almond production. With the exception of the product from a single producer, substantially all aflatoxin comes from ground and fine chopped manufacturing stock. The basis for this will be sought in further experiments.

The biochemistry involved in the browning of concealed damage almonds has been investigated. Preliminary results suggest that wetting of post harvest almonds activates the enzyme invertase. This, in turn, results in reducing sucrose to fructose and glucose. The reducing sugars then complex with basic amino acids or proteins. To this point, no heat is needed. When subsequently roasted, a Maillard reaction results in browning and off-flavors. Whether aflatoxin content is affected is not clear at this time. Possible reversal and spectral indicators (for sorting) of the pre-reaction are being investigated.

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IPM OF AFLATOXIN IN THE CORNBELT - INSECT ORIENTED FY 1996 RESULTS

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Resistance mechanisms

A commercial Bt hybrid was compared with a corresponding non-Bt hybrid for insect resistance of ears and thus potentially indirect resistance to ear mold. Expression of the Bt protein is high in green tissue and pollen, and much lower in other tissues. The Bt hybrid was free of first generation European corn borer (ECB) damage, while the non-Bt hybrid had 16% of plants damaged. Unfilled milk state ear tips had significant resistance to neonate ECBs in lab studies, producing 26-34% mortality and a 6-1OX reduction in growth rates of survivors (compared to < 1% mortality of the non-Bt hybrid) after 1 week. Milk stage Bt ears had a somewhat lower incidence of ECBs, and 9X fewer damaged kernels in ears where ECBs were found. Corn earworms occurred at low levels, and sap beetles were rare in milk stage ears. At harvest, incidence of insect damage that occurred at milk stage in the Bt hybrid was half that of the non-Bt hybrid, and half as many kernels were damaged when insects were present. The incidence of visible *Fusarium* mold was half that in Bt ears compared to non-Bt ears, and when mold was present the number of moldy kernels per ear was 2.5 fold less in the Bt hybrid compared to the non-Bt one. For the non-Bt hybrid, 9% of the ears had greater than 10 kernels molded with *Fusarium* per ear, while less than 1% of the Bt hybrid ears had this degree of mold. No sporulating A. *flavus* was noted in any ears. Despite the limited expression, the commercial Bt hybrid tested has the potential to have substantially reduced mold incidence compared to the non-Bt hybrid, and it is likely this will hold true for other hybrids engineered to express the Bt protein as well. As reported in 1995, expression throughout the plant (including kernels and silks) can nearly eliminate ear mold following ECB damage, but regulatory hurdles for approval are likely to be greater.

Transgenic *A. nidulans* expressing the restrictocin gene (from *A. restrictus)* had significantly increased resistance to sap beetle feeding, which further supports the role of this protein in insect resistance. A corn kernel protein was found to be toxic and/or have antifeedant activity at naturally occurring levels towards several kernel feeding insects. Targeted expression of the cloned gene in tissues of corn or other plants with low levels of the protein should help prevent insect-associated contamination of mycotoxigenic fungi. Corn earworms were found to be able to use some, but not all sterols as a nutrient source. These results suggest modification of sterol composition to one which is adverse to insects (as occurs in other plants), if not harmful to the plant, can reduce insect damaged and associated mycotoxin problems.

Transgenic tobacco plants overexpressing a tobacco anionic peroxidase showed some resistance to the silverleaf whitefly. Statistical regression was used to identify corn peroxidase isozymes associated with published levels of field resistance (Scott and Zummo) to *A. flavus* colonization. Correlations were sometimes greater than 90%, depending on year and inoculation method. One of the isozymes has been purified and partially sequenced. A CRADA has been signed with Pioneer Hi-Bred International to further explore the role of peroxidases in disease resistance.

Biological control

Enough of the sap beetle nematodes were collected that molted into males so that the species could be described; the proposed name of the new species is *Psammomermis nitiduensis* Poinar. Several hundred sap beetles infected with the nematodes were released at a site in Illinois that does not contain them in hopes they will become established and help control sap beetles in the area. The study with the release, recovery and identification of a strain of the insect pathogen *Beauveria bassiana* that is highly pathogenic on sap beetles has continued. Insects were reisolated from the field that were infected with the fungal pathogen and some initial PCR-based strain typing of the field isolates has been done.

Insecticide Encapsulation

In cooperation with Cerestar, U.S.A., Inc., granular 1% flour encapsulated malathion was aerially applied over a 6 acre area and compared with commercial 15% granular chlorpyrifos for insect control and ear mold reduction after commercial scouting established treatment was necessary. Sampling after 3 days indicated adverse affects of the chlorpyrifos on lady beetles, but the malathion had no effect on them. Leaf sampling after 6 and 12 days indicated approximately 30% control with the malathion and 85% with the chlorpyrifos. The malathion had no effect on lady beetles while the chlorpyrifos killed 60%. Examination of ears at harvest time indicated approximately 25% control of early European corn borer damage and visible *Fusarium* with the malathion granules, and about 20% control of each with the chlorpyrifos granules. The malathion granules reduced the number of ears that had more than 10 kernels molded with *Fusarium* by about 4 fold, while the chlorpyrifos reduced the number of these ears by about two fold. Scouting that targeted larger larvae than were targeted in past studies, and the use of somewhat larger granules than used in the past (which reduced coverage), may be the reason that lesser than desired control with the malathion granules was obtained. However, these limitations can be readily corrected.

Monitoring/prediction

At the request of an agricultural supply company (Gempler's) interested in licensing the patented insect trap (Dowd, Bartelt and Wicklow) and autoinoculator (Dowd and Vega), field trials to validate a modified design were successfully completed.

From regression modeling with available weather and aflatoxin levels previously reported in the corn belt, temperature and rainfall were found to interact prior to midsummer to promote conditions conducive to later aflatoxin formation (presumably due to promotion of conditions favoring growth of *A. flavus*). Early predictability of conditions conducive to aflatoxin

formation can allow for "rescue" treatments during ear fill (such as insect or fungal control) to help reduce aflatoxin levels. The predictive model has been partially written.

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EFFECTS OF THE SOUTHWESTERN CORN BORER ON AFLATOXIN CONTAMINATION

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The effect of the southwestern corn borer (SWCB) on aflatoxin contamination and *Aspergillus flavus* kernel infection in corn was investigated in field experiments in 1995 and 1996. Inoculation techniques and placement of the two pests were studied to determine their effect on the aflatoxin contamination. In 1995 when *A. flavus* was applied to the silks in a spray and SWCB in corn cob grits was placed in the leaf axil at the top ear, aflatoxin contamination was higher in plants infested with both pests. Aflatoxin levels in plants treated with *A. flavus* alone were relatively low. Kernel infection was highest in all hybrids treated with SWCB and *A. flavus*. In 1996, aflatoxin levels were relatively low in all plants. SWCB treatments had no apparent-effect on aflatoxin contamination. Differences in the effect of SWCB on aflatoxin contamination may be due to higher natural infection by *A. flavus* in 1995 than in 1996. In another experiment, SWCB and *A. flavus* were applied in corn cob grits to plants at the late whorl stage. Aflatoxin levels were highest in several hybrids treated with SWCB alone. The combination treatment with both pests was not different from plants treated only with *A. flavus*. An experiment was conducted in 1996 where both pests were applied in corn cob grits to the silks of ten hybrids. Aflatoxin levels were much higher in plants treated with both pests in comparison to plants treated with only *A. flavus* or not treated with any pest. Hybrids made from resistant genotypes had high levels of aflatoxin contamination when SWCB was included with *A. flavus*, but low levels of aflatoxin when the insects were absent. These studies indicate that SWCB can substantially increase aflatoxin levels. Also, inoculation methods and placement of the pests on plants can have a dramatic effect on aflatoxin contamination.

POSTER PRESENTATIONS

SOURCES OF AFLATOXIN VARIABILITY IN PISTACHIOS

Noreen Mahoney and Susan Rodriguez, USDA, ARS, Western Regional Research Center, Albany, CA.

The incidence of aflatoxin contamination in pistachios is low, but aflatoxin levels can be quite variable and high levels can develop in a small percentage of nuts. A single pistachio with 60μg aflatoxin can contaminate an otherwise aflatoxin-free 10 lb. test lot at the FDA action level of 20 ppb. Thus, it is of interest to determine why certain nuts are susceptible to developing high levels of aflatoxin.

The pistachio fruit consists of an edible kernel and seed coat encased in a hardened shell, all of which are surrounded by a fleshy hull which is removed during processing. Pistachios fruit tissues, including the hull, seed coat, and kernel, all contribute to aflatoxin variability. Intact kernels and hulls are protected from *A. flavus* germination and/or colonization by the waxy cuticular layer on the surface of these tissues. Damaged kernels and hulls are readily colonized by *A. flavus*; however, no aflatoxin is produced in the hulls and variable levels of aflatoxin are produced in the kernels. Seed coat can delay *A. flavus* germination and/or colonization and also contributes to variable aflatoxin production.

It is likely that endogenous chemical factors are responsible for the inhibition and variability of aflatoxin production in these tissues. Pistachio hulls, kernels, and seed coats were extracted with increasingly polar solvents and the extracts tested for aflatoxin inhibitory activity. Hydrolyzable tannins isolated from hulls incorporated into media at 0.1% inhibited aflatoxin production by 99%. Seed coat tanning also inhibited aflatoxin production. Kernel extracts are being further fractionated to identify inhibitory compounds.

Aflatoxin Levels of In-hull and Hulled Individually Inoculated Pistachios

	Aflatoxin B1 in 50 Kernels	
	Range (µg/kernel)	Average (µg/kernel)
In-hull		
Unwounded	0-11	0.7
Wounded	0-14	1.8
Hulled With seed coat		
Unwounded	0 - 460	15
Wounded	1.3 - 890	290
Hulled Without seed coat		
Unwounded	0	0
Wounded	30 - 710	290

THE STEM END: A POTENTIAL ENTRY POINT FOR *A. FLAVUS* IN CLOSED SHELL PISTACHIOS

Noreen Mahoney and Russell Molyneux, USDA, ARS, Western Regional Research Center, Albany, CA.

Preharvest aflatoxin contamination in pistachios primarily occurs in insect damaged, early split pistachios. In early split pistachios, the hull and shell split together, exposing the kernel to insect and fungal attack. *A. flavus*, being a weak plant pathogen, can more easily colonize kernels with insect damage. However, aflatoxin has also been detected in closed shell pistachios. In these pistachios the shell never splits, thus never exposing the kernel to insect or fungal damage. This study demonstrates that *A. flavus* can colonize the nutmeat through the closed shell stem end. Seventy-three percent of fresh closed shell pistachios which were inoculated with 200 spores *A. flavus* on the stem end developed visible *A. flavus* contamination on the stem end. Fifty-four percent of these kernels developed aflatoxin contamination, with an average of 56 μg/kernel. Some kernels developed levels as high as 700 μg/kernel. Aflatoxin was not detected in any closed shell pistachios with non-inoculated stem ends.

Shell stem ends could become inoculated with *A. flavus* spores during processing. After the hulls are removed, the pistachios are transferred to a flotation bath. *A. flavus* spores in the flotation bath can inoculate the stem ends of pistachios. In a simulated flotation bath, 95% of closed shell pistachios placed in a flotation bath containing 100 spores/ml developed stem end colonization. Closed shell pistachios inoculated in the flotation bath can develop postharvest aflatoxin contamination with any delay or irregularities in the drying process.

Aflatoxin Levels in Closed Shell Pistachios with Stem Ends Inoculated with *A. flavus*

Percentage of Kernels	Aflatoxin Levels (μg/kernel)
46%	0
10%	<1
26%	1 to 60
18%	>60

DATABASE AND ALGORITHMS FOR X-RAY SORTING OF INSECT DAMAGED PISTACHIO NUTS. AN OVERVIEW.

P. M. Keagy[1] and D. P. Casasent[2], [1]USDA, ARS, Western Regional Research Center, Albany, CA; and [2]Dept. of Electrical and Computer Engineering, Carnegie Mellon University, Pittsburgh, PA.

The US now produces 34% of the world's pistachio nuts and is second only to Iran in total production. International markets are setting strict limits on aflatoxin (a potent natural carcinogen) contamination and US producers are seeking ways to assure that the aflatoxin content of their products is as low as possible. Previous work by Sommer indicated that no aflatoxin was found in healthy nuts with intact hulls before harvest. Nuts with hulls which split before harvest were at risk for aflatoxin contamination while infestation by naval orange worm increased the probability of aflatoxin contamination. Currently, insect infested nuts are removed from the processing line when other defects are present or by manual inspectors when external evidence of infestation is present. US standards for grades of pistachio nuts restrict insect damage to 1-3% (by weight) of nuts. A nondestructive method of inspection for insect damage is not currently available. This poster presents an overview of three stages in the development of automated x-ray sorting for insect damages pistachios. This work is the result of collaboration between Western Regional Research Center, ARS, USDA, Albany, CA and the Dept of Electrical and Computer Engineering, Carnegie Melon University, Pittsburgh, PA. Full proceedings papers will be available in SPIE vol. 2907 Nov. 1996, papers 20, 21, and 22.

EXPANDED IMAGE DATABASE OF PISTACHIO X-RAY IMAGES AND CLASSIFICATION BY CONVENTIONAL METHODS

P. M. Keagy[1], T. F. Schatzki[1], L. Le[1], D. Casasent[2] and D. Weber[2], [1]USDA, ARS, Western Regional Research Center, Albany, CA; and [2]Carnegie Mellon University, Pittsburgh, PA.

In order to develop sorting methods for insect damaged pistachio nuts, a large data set of pistachio x-ray images (6759 nuts) was created. Both film and linescan sensor images were acquired, nuts dissected and internal conditions coded using the U.S. Grade standards and definitions for pistachios. A subset of 1199 good and 686 insect damaged nuts was used to calculate and test discriminant functions. Statistical parameters of image histograms were evaluated for inclusion by forward stepwise discrimination. Using three variables in the discriminant function, 89% of test set nuts were correctly identified. Comparable data for 6 human subjects ranged from 67 to 92%. If the loss of good nuts is held to 1% by requiring a high probability to discard a nut as insect damaged, approximately half of the insect damage present in clean pistachio nuts may be detected and removed by x-ray inspection.

DETECTION AND SEGMENTATION OF MULTIPLE TOUCHING PRODUCT INSPECTION ITEMS

D. Casasent, Ashit Talukder, Westley Cox, Hsuan-Ting Chang and David Weber, Dept. of Electrical and Computer Engineering, Laboratory for Optical Data Processing, Carnegie Mellon University, Pittsburgh, PA.

X-ray images of pistachio nuts on conveyor trays for product inspection are considered. The first step in such a processor is to locate each individual item and place it in a separate file for input to a classifier to determine the quality of each nut. This paper considers new techniques to: detect each item (each nut can be in any orientation, we employ new rotation-invariant filters to locate each item independent of its orientation), produce separate image files for each item (a new blob coloring algorithm provides this for isolated (non-touching) input items), segmentation to provide separate image files for touching or overlapping input items (we use a morphological watershed transform to achieve this), and morphological processing to remove the shell and produce an image of only the nutmeat. Each of these operations and algorithms are detailed and quantitative data for each are presented for the X-ray image nut inspection problem noted. These techniques of general use in many different product inspection problems in agriculture and other areas.

NEUTRAL NET CLASSIFICATION OF X-RAY PISTACHIO NUT DATA

David Casasent[1], Michael A. Sipe[1], Thomas F. Schatzki[2], Pamela M. Keagy[2] and Lan Chau Le[2], [1]Carnegie Mellon University, Pittsburgh, PA; and [2]USDA, ARS, Western Regional Research Center, Albany, CA.

Classification results for agricultural products are presented using a new neural network. This neural network inherently produces higher-order decision surfaces. It uses new techniques to select the number of hidden layer neurons and adaptive algorithms that avoid other such ad hoc parameter selection problems: it allows selection of the best classifier parameters without the need to analyze the test set results. The agriculture case study considered is the inspection and classification of pistachio nuts using x-ray imagery. Present inspection techniques cannot provide good rejection of worm damaged nuts without rejecting too many good nuts. X-ray imagery has the potential to provide 100% inspection of such agricultural products in real time. Only preliminary results are presented but these indicate the potential to reduce major defects to 2% of the crop with I% of good nuts rejected. Future image processing techniques that should provide better features to improve performance and allow inspection of a larger variety of nuts are noted. These techniques and variations of them have uses in a number of other agricultural product inspection problems.

THE POSSIBILITIES ON REDUCTION OF AFLATOXIN CONTAMINATION IN DRIED FIGS THROUGH CONTROLLING AFLATOXIGENIC FUNGI BY FUNGICIDES

N. Tosun and N. Deten, Department of Plant Protection, Faculty of Agriculture, Ege University, Bornova 35100, Izmir, Turkey.

The Aegean region provides more than 50% of fig production in the world. Particularly firm ripe and shriveled figs are the most vulnerable for colonization of aflatoxigenic fungi, *Aspergillus flavus* and *A. parasiticus*. The higher population of the fungi in orchard soil, the higher chance of aflatoxin development in figs. Inhibition of fungal population around tree canopy and on top soil under the trees where the figs drop seem to be an easy way to reduce infection by the fungi.

In this paper, cultures of *A. flavus/parasiticus* collected from orchards and processing houses were evaluated for their aflatoxin producing ability. Then, seven fungicides, namely, copper oxychloride, captan, thiram, chlorothalonil, mancozeb, benomyl, and prochloraz were tested first in laboratory and in pot conditions for effectiveness against 14 selected isolates. Prochloraz at 3 μg/ml concentration was found to be the most effective against the fungi, both in vitro and pot experiments as to ED50 and MIC values followed by benomyl. However, no fungicide suppressed significantly the growth of the fungi in artificially infested soil. Monthly variation in population of aflatoxigenic fungi in fig orchard soil and conidia dispersal times were determined to make proper application strategies. The highest population density was found in the average of 700 μg/g in March and April following heavy rain in the orchard. Significant decreases were observed after ploughing the soil in the orchards (levels were 25 μg/g). Conidia dispersal was first observed in April after irrigation in 1995. All fungicide treatments in fig orchards had been effective enough in reducing the source of fungal spores and lower the level and frequency of aflatoxin contamination. Furthermore, high aflatoxin concentrations were analyzed from untreated dried figs which also had more spores. Although the numbers of application are high during the studies, tree application before fruiting, treatments of ground surface before the fig drop under the trees and drying place should be useful for decreasing invasion of not only aflatoxigenic fungi and minimizing aflatoxins, but also other fungal pathogens of figs such as Fusarium, Phomopsis and Alternaria.

For Panel Discussions, Panelists Combined the following Two Workshop Discussions:

MOLECULAR MECHANISMS GOVERNING AFLATOXIN PATHWAY AND GENE EXPRESSION

and

POTENTIAL USE OF NATIVE PLANT COMPOUNDS FOR THE PREVENTION OF AFLATOXIN CONTAMINATION

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Utilizing Knowledge of Aflatoxin Gene Expression and Native Plant Compounds for Host Plant Resistance Enhancement Strategies.

PANEL MEMBERS: Gary Payne (Chair), Deepak Bhatnagar, Bruce Campbell, Fun Sun Chu, John Linz, J. E. Mellon and Bob Norton

SUMMARY OF PRESENTATIONS: The use of fungal gene reporter constructs to measure fungal growth and aflatoxin biosynthesis, and the development of sensitive assays, has lead to an explosion of research in the area of native plant compounds for host plant resistance. Compounds inhibitory to aflatoxin biosynthesis have been identified in corn, cottonseed, peanut, and tree nuts. These compounds include proteins, anthocyanins, carotenoids, plant volatiles, and a complex carbohydrate. The strategies employed are the inhibition of aflatoxin formation per se, the inhibition of fungal growth, the inhibition of insect feeding, and the use of sex pheromones to disrupt insect mating. There also has been significant progress on the characterization of aflatoxin biosynthesis this past year. Several labs have now confirmed the link between development and aflatoxin biosynthesis, and they are beginning to understand the common regulatory elements of the two. Studies have shown that the *nor1* and *ver1* promoters are subject to temporal and spatial expression, and that the highest levels of promoter activity are associated with the conidiophores and, in particular, in vesicles during early stages of development. Information gained from the above studies may lead to an understanding of the role of aflatoxin in biology of the fungus. Recent research also has lead to the identification of a gene, *aflJ*, whose disruption results in strains that do not accumulate pathway intermediates. Thus, this gene is a good target site for the inhibition of aflatoxin biosynthesis. The productivity in the research area "Utilizing Knowledge of Aflatoxin Gene Expression and Native Plant Compounds for Host Plant Resistance Enhancement Strategies" is evident by the number of posters presented on this subject. Of the 39 posters presented at this meeting, 60% addressed research findings in this area. Below is a summary of the accomplishments and applications of the research from this area.

Accomplishments

1. The biosynthetic pathway of aflatoxin is becoming well understood, and models are being developed to describe the regulatory control of the pathway.
2. A relationship between fungal development and aflatoxin biosynthesis has been established.
3. Knowledge of the aflatoxin biosynthetic pathway and its regulation has lead to the development of gene reporter constructs that have been used to identify inhibitory compounds from native plants.
4. Laboratory-based assays have been developed to identify inhibitory compounds.
5. Plant compounds have been identified that inhibit aflatoxin biosynthesis.
6. Plant compounds have been identified that inhibit fungal growth.
7. Information gained from this group has broad application across commodities.

Application of the Information Gained

1. Well characterized biocontrol strains can be engineered.

Our understanding of aflatoxin biosynthesis and its regulation will allow the targeting of specific sites within the fungus. Further, our understanding of the relationship between fungal development and aflatoxin biosynthesis will allow the specific targeting of sites that inhibit aflatoxin biosynthesis but do not affect the survivability of the fungus.

2. Potential target sites for inhibition of aflatoxin biosynthesis can be identified.

Our understanding of aflatoxin biosynthesis and regulation allows the identification of specific sites that can be targeted by compounds inhibitory to aflatoxin biosynthesis. It also may be possible to predict which sites are less likely to cause the fungus to develop resistance to the toxic compound. The availability of cloned pathway genes allows the development of reporter constructs that can be used to screen potential inhibitory compounds.

3. Natural plant compounds associated with host resistance can be readily identified in laboratory assays.

A number of sensitive assays have been developed that allow for the screening of plants in the laboratory or field for the presence of inhibitory compounds. Many of these assays are based on reporter constructs or the accumulation of a pathway intermediate. Data presented at this meeting has shown that such assays work.

4. Mechanisms of host resistance can be determined.

By using gene reporter constructs of pathway genes it is now possible to identify the mechanisms and sites of action of various inhibitory compounds. This is very important because it allows breeders to select for multiple forms of resistance.

5. Plants with multiple forms of resistance can be developed.

The identification of chemicals associated with resistance provides a chemical marker to aid in marker-assisted breeding. By associating chemical marker with genetic markers, plant breeders can identify genetic loci in plants associated with resistance and readily select plants with multiple forms of resistance.

6. Resistance can be developed by breeding or by engineering transgenic plants.

The identification of chemical compounds associated with resistance allows the development of two control strategies. They can be used as described above as chemical markers in marker-assisted plant breeding or they can be used in the development of transgenic plants. The gene

coding for a toxic compound in corn, for example, could be used to develop transgenic resistance in any of the other plant species.

7. Molecular based assays allow rapid screening of transgenic plants for expression of inhibitory compounds.

Reporter gene constructs are now available to monitor fungal growth and aflatoxin formation. The use of these constructs allow the identification of resistance in breeding material and in transgenic plants.

SUMMARY OF PANEL DISCUSSION: A topic of discussion was the relationship between fungal growth (fungal mass) and aflatoxin production and between fungal development (reproductory and overwintering structures, for examples spores and sclerotia, respectively) and aflatoxin production. There is a large body of evidence to show that aflatoxin accumulation is not necessarily correlated with the accumulation of fungal mass. However, there is increasing evidence that aflatoxin production and fungal development are related, and that the two may share common regulatory genes. Even though the two pathways appear to have common regulatory genes, it is clear that two separate pathways exist for development and aflatoxin biosynthesis. A point was raised as to the benefit of understanding development if the ultimate goal is to block aflatoxin formation. It was argued that for the development of biocontrol strains it is important to know the effect of loss of aflatoxin production on the development of the fungus. For example, while it is desirable to have a strain that does not produce any of the pathway intermediates, it is not desirable to delete genes that may influence the formation of sclerotia or affect the survival of the fungus. A question was raised as to the possible number of inhibitory compounds in plants. It was agreed that there is a diverse germplasm to be screened for resistance. One unknown in this area is the concentration of the inhibitory compounds in the part of the seeds that is attacked by *A. flavus*. In corn, for example, it is thought to be important that the embryo contain a sufficient concentration of the inhibitory compound. Less information is available on potential stimulatory compounds in seeds, but a poster at the meeting showed that amylase production by the fungus may be important for the release of stimulatory sugars. The session ended with the comment that we now have the molecular tools to begin melding the mapping of crop resistance genes with the presence of inhibitory compounds in the plants.

PLATFORM PRESENTATIONS

Molecular Mechanisms Governing Aflatoxin Pathway and Gene Expression

PRODUCTS OF THE LIPOXYGENASE PATHWAY IN PLANTS THAT REGULATE AFLATOXIN BIOSYNTHESIS

Nancy P. Keller, Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX.

The carcinogenic compounds aflatoxin (AF) and sterigmatocystin (ST) are products of the same secondary metabolic pathway and are found in several fungal genera, most notably the genus *Aspergillus*. In addition to the serious health risk these compounds pose, continual *AF/ST* contamination of a vast array of agricultural products has seriously impacted agricultural and economic policies worldwide. The research on the biosynthesis and regulation of these toxic secondary metabolites in fungi together with studies on the interaction between plants and fungi could provide basic understanding that would lead to the design of novel targeted control strategies. In this regard, the biotechnological manipulation of plant compounds with antifungal properties is currently gaining relevance as a means to eliminate plant diseases. Recently our lab found that the plant metabolite 13-hydroperoxy linoleic acid 13(S)-HPODE, produced by soybean lipoxygenase 1 reduced and/or inhibited the biosynthesis of these carcinogenic fungal compounds (see posters). Our interest is two-fold, to:

(1) determine if 13(S)-HPODE acts to inhibit AF production in vivo by transforming two cultivars of peanut (Georgia Runner and Florunner) with the soybean lipoxygenase1 gene, LOX1.

(2) elucidate the mechanism(s) through which 13(S)-HPODE inhibits the AF/ST biosynthetic pathway.

Our posters (see Burow et al. and Calvo et al. in this issue) describe our progress on these goals. We currently have 26 and 50 putative LOX1 transformants of Georgia Runner and Florunner, respectively. If we find they contain the LOX1 gene, we will test them for relative resistance to aflatoxin contamination (Burow et al.). With regard to mechanism of how 13(S)-HPODE effects the AF/ST pathway, our initial findings suggest that it is through acting on the development of the fungus. We believe that 13(S)-HPODE may act to mimic or inhibit an *Aspergillus* pheromone as we find that *A. nidulans* LOX1 transformants are impaired in sexual development (Calvo et al.). This change in development may be linked to AF/ST biosynthesis in the aspergilli.

IMMUNOCHEMICAL STUDIES OF THE ENZYMES OF AFLATOXIN BIOSYNTHESIS

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Attempts were made to produce both monoclonal (Mab) and polyclonal (Pab) antibodies against several key enzymes and regulatory proteins involved in aflatoxin (AF) biosynthesis, i.e., sterigmatocystin (ST) methyltransferase (ST-MTFase), norsolorinic acid reductase (NSR), Ver-1, polyketide syntase (PKS) and *afl-R* protein. In the last few years, we have successfully obtained both Mab and Pab for NSR and ST-MT-transferase. Effective enzyme-linked immunosorbent assays (ELISA) were established for these enzymes and subsequently used in a study elucidating the role of these enzymes in AFB1 formation. During 1995-96, much of our effort was devoted to the production and characterization of antibodies against *afl-R* protein (AFLR), the regulatory protein involved in the activation of aflatoxin biosynthesis pathway.

A. Research Progress:

1. Production and characterization of antibodies against AFLR protein: Polyclonal antibodies against AFLR, the *aflR* gene product of *Aspergillus flavus* and *A. parasiticus,* were generated by immunizing a rabbit with the *Escherichia coli*-expressed recombinant AFLR protein of *A. flavus*. Immunoblot analysis revealed that the antibodies reacted primarily with the recombinant AFLR protein of *A. flavus* or of *A. parasiticus,* as well as with native 47 kDa AFLR in *A. parasiticus*. Accumulation of AFLR and aflatoxin in *A. flavus* and *A. parasiticus* was demonstrated in the glucose mineral salts medium (GMS), which supports aflatoxin formation, when the fungi were first grown in the peptone mineral salts medium (PMS), which does not support aflatoxin formation. Western blot analysis revealed that the 47 kDa AFLR protein was present in the cell-free fungal extracts of *A. parasiticus* NRRL 2999 grown in GMS medium, but not in the culture grown initially in PMS medium. The AFLR protein was absent in the non-aflatoxigenic *Penicillium* and *Fusarium* species grown in GMS medium. The induction pattern of AFLR in aflatoxigenic *A. parasiticus* was regulated by carbon source, and correlated well with that of aflatoxin accumulation in the culture filtrate.

2. Immunochemical analysis of regulatory expression of AFLR protein and its relationship with aflatoxin biosynthesis: The regulatory effects of various environmental and nutritional conditions, including temperature, oxygen, carbon source, nitrogen source and zinc availability, on the expression of AFLR protein and aflatoxin production in *A. parasiticus* NRRL 2999 were investigated. AFB_1 production occurred in GMS medium at 29°C but not at 37°C. Western blot analysis with anti-AFLR antibodies showed that the level of AFLR in 37°C GMS culture was 4-fold lower than that in 29°C GMS culture. Neither AFB_1 nor AFLR was detected in the 29°C culture with PMS medium. Accumulations of AFLR protein and aflatoxin were maximal in the shaken liquid culture, but were depressed in the static culture with GMS medium. Compared with the AFB_1 concentration in regular GMS cultures, aflatoxin formation in GMS medium deficient in zinc (GMS minus Zn) or with sodium nitrate (GMS plus $NaNO_3$) as the sole nitrogen source was suppressed 85% and 93%, respectively. Immunoblot analysis also indicated that AFLR expression in the GMS minus Zn culture and the GMS plus $NaNO_3$ culture was 4-fold and 8.3-fold less than that in GMS medium.

However, AFB_1 concentration and AFLR levels in GMS medium with the ammonium nitrate as the only nitrogen source were slightly higher than those in regular GMS culture. Our results suggested that expression of AFLR protein was regulated by environmental factors and nutritional factors; high temperature (i.e., 37°C), limited supply of oxygen, inappropriate carbon source and nitrogen source, and insufficient zinc ion in medium may negatively regulate AFLR expression and then lead to the suppression of AFB_1 production.

B. Implication of research data: Over the years, we have collaborated with scientists in USDA and several universities (U. of Wisconsin, North Carolina State, Michigan State and Texas A & M) who are actively involved in characterizing the genes in the aflatoxin biosynthesis. Antibodies have played an important role in the successful cloning of several genes for key enzymes in the aflatoxin biosynthetic pathway. With the availability of antibodies, specific ELISA methods for these enzymes have been established. These methods have proved to be effective in differentiating the toxin producing fungi from non-producers. Using different immunochemical methods, we have demonstrated that formation of aflatoxin is highly regulated by AFLR protein, and the expression of the AFLR gene is regulated by various nutritional and environmental factors. Thus, immunochemical analysis proved to be an effective tool in identifying various factors and control agents that could control aflatoxin formation. In addition, these antibodies have been used in immunoaffinity chromatography as a simple method for purification of these enzymes. All these technologies could be transferred to other scientists to facilitate further work on the control of aflatoxins in field crops.

C. Publications:

Yu, J., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Keller, N.P. and Chu, F. S. 1993. Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an O-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 3564-3571.

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IDENTIFICATION OF CRITICAL FACTORS REGULATING AFLATOXIN BIOSYNTHESIS

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Our work continues to focus on the regulation of expression of several key genes involved in aflatoxin B_1 (AFB_1) biosynthesis in *Aspergillus parasiticus* including *nor*-1 (Chang et al., 1992; Trail et al., 1994), *ver*- 1A (Skory et al., 1992; Skory et al., 1993; Liang et al., 1996A), *fas*-1A (Mahanti et al., 1996). *fas-2A* (Trail et al., 1995; Neely et al., 1996), and *pksA* (Trail et al., 1995). The work can be subdivided into 3 key projects: 1) **promoter structure/function**; 2) **gene structure/function**; and 3) **protein localization**. Posters summarizing results in project I (Miller and Rarick; Wilson) and 2 (Neely and Wood) are being presented and abstracts included in the Aflatoxin Elimination Conference (AEC) proceedings. In addition, Dr. Frances Trail now a faculty member in Botany and Plant Pathology at Michigan State University, continues her collaborative efforts on aflatoxin biosynthesis with our lab, but has also initiated independent work on natural resistance to aflatoxin in peanuts. Her work is being presented in poster format at this meeting. An abstract is included in the AEC proceedings.

Project 1: Promoter structure/function. Currently, effort is focused on *nor-1* and *ver*-1A, but the work eventually will include studies on *fas*-1A and *fas*-2A when the promoter region for these divergently transcribed genes is more clearly defined. The polymerase chain reaction was utilized to divide *nor*-1 and *ver*-1 promoters into 3 subfragments of a practical size for gel mobility shift analysis. Proteins from nuclei obtained from cells grown under aflatoxin inducing conditions (GMS media) shifted promoter fragments from both promoters, whereas proteins from nuclei in noninduced cells (PMS media) did not. The specificity of the shift of one fragment was clearly established (*nor*-R) using competition experiments while the specificity of other shifts is under study (Miller et al., 1996). These data support the hypothesis that promoter function is regulated by at least 1 positively acting transacting factor. DNA footprint analysis is underway to clearly define cis- acting sites in *nor*-1 and *ver*-1 promoters.

A parallel approach is also being utilized to measure promoter function *in vivo*. Reporter constructs, *nor*-1/GUS and *ver*-1/GUS, were transformed into *A. parasiticus*. GUS (uidA, encodes β-glucuronidase) activity was measured at appropriate time points in cells growing in liquid and solid media. The data confirmed that aflatoxin biosynthesis is regulated in part at the transcriptional level and that reporters can be utilized to map cis-acting sites in these promoters via *in vitro* mutagenesis and functional assay *in vivo*. The data also suggested several other interesting things: 1) The *nor*-1 and *ver*-1 promoters are subject to temporal and spatial regulation. (Liang et al., 1996B; Zhou, 1996); 2) Although promoter activity can be detected in almost all cells, highest levels of activity are associated with conidiophores, and in particular in vesicles during early stages of development (Liang, 1996C; Zhou, 1996). Because of the association between expression and development, it is likely that mechanisms of regulation differ when cells are grown in liquid media (conidiophore development is inhibited) and on solid media (conidiophore development proceeds); 3) The location of genes within the cluster may be important for normal regulation. When the reporter construct integrates into the chromosome within the aflatoxin gene cluster, GUS expression is 500 fold greater than

when the reporter integrates at *niaD* or at 2 other unmapped sites in the chromosome (Liang et al., 1996A; Wilson et al., 1996). Work is continuing on items 2 and 3 to help confirm and expand on these observations.

Project 2: Gene structure/function. Gene structure/function. As reported previously, disruption of *nor*-1 (Trail et al., 1994), *ver*-1A (Liang et al., 1996A, *fas*-1A (Mahanti et al., 1996), and pksA (Trail et al, 1995) resulted in important insights into the function of these genes in the AFB_1 pathway. For example, using disrupted mutants of *pks*A and *fas*-1A, Watanbe et al. (1996) clearly established the role of these genes in polyketide backbone synthesis. The data also suggested that some form of "channeling" may occur in the early steps in AFB_1 biosynthesis in *A. parasiticus* which could result from direct protein interaction or from an activation/transport mechanism which allows the product of one enzyme to be efficiently utilized as a substrate by the subsequent enzyme activity. Some of these knockout strains have the potential to be utilized as biocontrol agents for reduction of AFB_1 on crops. Work is continuing on the disruption of *fas*-2A and gene3 (Neely et al., 1996). Screening is underway for knockouts of these 2 genes--some promising transformants have been identified.

Project 3: Protein localization. Polyclonal antibodies, raised to Nor-1 and Ver-1 fusion proteins synthesized in *Escherichia coli* (Liang et al., 1996B; Zhou and Linz, 1994), were shown to specifically recognize the fungal Nor-1 and Ver-1 proteins as well as their *E. coli* counterparts. Western blot analysis of protein samples obtained at appropriate times from cells grown in liquid media and on solid media confirmed that Nor-1 and Ver-1 proteins accumulate with similar temporal and spatial patterns as AFB_1 and the transcripts that encode these activities (Liang et al., 1996B, Zhou, 1996). Differential centrifugation of extracts of fungal cells grown under AFB_1 inducing conditions demonstrated that the Nor-1 protein was primarily associated with the cytoplasmic fraction (approx. 97%). A small quantity of the protein (approx. 3%) was associated with "structures" or "particles" that pelleted together with lysosomes and microbodies as demonstrated by analysis of key marker enzymes in these cell fractions (Zhou, 1996). A significant fraction of Ver-1 protein (approx. 14%) pelleted in the same fraction as mitochondria suggesting an association of this activity with a "particle" or "structure" of uncharacterized composition. Immunolocalization studies were performed with fluorescent labeled antibodies to identify Nor-1 and Ver-1 proteins *in situ* in cells grown on solid media (Liang, 1996C; Zhou 1996). These data confirmed that Nor-1 is primarily an enzyme activity associated with the cytoplasm but Ver-1 is strongly associated with "particles" or "structures" of uncharacterized composition (consistent with data derived from differential centrifugation). Confocal laser microscopy of these "particles" demonstrated that they are contained within the mycelium of the fungus. Immunofluorescence microscopy also suggested that the Nor-1 and Ver-1 proteins are produced in most cells in a growing culture but that the highest quantities of these proteins (both free and in "particles") are associated with developing conidiophores consistent with data obtained using *nor*-1/GUS and *ver*-1/GUS reporter constructs. Interestingly, the Nor-1 protein was detected at high levels in immature conidia but the quantity declined as the spores matured. Together these data suggest a strong association between AFB_1 synthesis and spore development and perhaps provide important clues to at least one biological function for this mycotoxin.

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CAN WE SIMULTANEOUSLY AFFECT FUNGAL DEVELOPMENT AND AFLATOXIN BIOSYNTHESIS?

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Aflatoxin synthesis in *Aspergillus parasiticus* and *A. flavus* is considered to be a "secondary" metabolic process and has no obvious physiological role in growth and primary metabolism of the organism. As yet, there is no confirmed biological role of aflatoxin in the ecological survival of the fungal organisms. Because aflatoxins are toxic to certain potential competitor microbes in the ecosystem and insect pests of crops inhabitated by the aspergilli, a survival benefit to toxigenic fungi is implied. It should be noted, however, that aflatoxin *per se* is a poor antibiotic.

However, it has been recently determined that aflatoxin synthesis occurs in a very complex and highly organized manner. The genes involved are organized systematically in a cluster on one of the fungal chromosomes, suggesting that aflatoxins may have some significant function in the life cycle or survival of the fungus. Conidia (asexual spores) and sclerotia (resting/survival structures) are specialized structures used by the fungus for dissemination and survival, respectively.

Recent observations from our lab and those from J. Linz' lab have suggested that normal sclerotia development may be correlated to normal aflatoxin production. A genetic relationship between aflatoxin biosynthesis and sclerotial development was examined in *A. parasiticus* SRRC 2043. This strain, which accumulates 0-methylsterigmatocystin, demonstrated an over expression of the aflatoxin pathway genes, *aflR* and *aflJ*, when transformed with additional copies of these genes (*aflR* is a pathway regulatory gene and *aflJ* has been demonstrated by Payne and coworkers to affect aflatoxin production). Elevated levels of aflatoxin intermediates were produced by introduction of the extra copies of either *aflR* or *aflR* plus *aflJ* in transformants, but not by the transformed *aflJ* alone. The number of sclerotia produced in *aflR* and in *aflR* plus *aflJ* transformants on PDA plates increased significantly but no change was observed in the *aflJ* transformant. This increase in the number of sclerotia was concomitant with a decrease in the sclerotial size. However, the sclerotial number of the *aflR* plus *aflJ* was substantially decreased on CZ plates. An increase in the production of aflatoxin intermediates resulted in a change in sclerotial morphology as well; the regular round/oval shape was modified to elongated/bullet shape depending on the medium used. Scanning electron micrographs showed that the sclerotia of the *aflR* plus *aflJ* transformant was not as compact as that observed for the wild-type strain (Figure--see Chang, et al.). These results suggest a regulatory association between sclerotial morphogenesis, aflatoxin biosynthesis and possibly other cellular processes.

In other studies, we have demonstrated that alterations in the regulation of aflatoxin biosynthesis are correlated with alterations in the conidial morphology of the fungus. Six non-aflatoxigenic variants of *A. parasiticus*, isolated after 5 to 12 serial transfers of non-sporulating

mycelia and designated *sec-*, were characterized morphologically by electron microscopy, biochemically by biotransformation studies with aflatoxin precursors and genetically by Northern hybridization analysis of aflatoxin biosynthetic gene transcripts. Scanning electron micrographs demonstrated that, compared to the parental aflatoxigenic *sec+* forms, the variant *sec-* forms had an abundance of vegetative mycelia, a significantly reduced number of conidiophores and conidia, and the presence of abnormal metullae (Figure). All *sec+* forms but none of the *sec-* forms showed bioconversion of sterigmatocystin (ST) to aflatoxins. Northern blots probed with pathway genes demonstrated lack of expression of both the aflatoxin biosynthetic pathway structural *(nor-1, omt-1)* and regulatory (*aflR*) genes in *sec-* form may be the result of some regulatory abnormality, which is also associated with abnormal fungal morphology. Therefore, the possibility exists that the regulations of aflatoxin synthesis and fungal development may be interlinked.

The question therefore is that can we through breeding and genetic engineering programs affect not only aflatoxin production by the fungus, but also its long term survival by affecting fungal development? And, can this be achieved using plant metabolites?

When aflatoxigenic strains of *A. flavus* were grown in the presence of specific cotton-leaf or maize volatiles, increases or decreases in aflatoxin production as well as variations in fungal growth were observed. In some cases, growth was not significantly affected while aflatoxin biosynthesis was markedly inhibited. For this purpose, four metabolites from almost 50 cottonleaf volatiles were selected for analysis, including two alcohols (3-methyl- 1-butanol and 1-nonanol) and two terpenes (limonene and camphene). These compounds had significant affects on either fungal growth or aflatoxin production. The effects of volatile exposure at 0, 10, 25, 50, and 100 l each of 3-methyl-l-butanol (butanol), nonanol, limonene, and camphene were evaluated. Butanol treated samples exhibited a decrease in radial growth that was directly proportional to butanol dosage. However, aflatoxin production was enhanced. In addition, butanol treatment resulted in a loss of pigmentation that was accompanied by a marked decrease in sporulation. Limonene and camphene-treated samples yielded negligible differences in radial growth and morphology when compared to control. But significant reduction in aflatoxin production was observed with limonene and not with camphene. Samples grown in the presence of all doses of nonanol demonstrated uniquely aerial hyphae, and radial growth was inhibited by 50 percent at 100 l of nonanol, but no significant impact on aflatoxin production was observed.

These results suggest that plant metabolites vary in their effects on aflatoxin biosynthesis and fungal development. Therefore, regulation of aflatoxin biosynthesis by these metabolites should be studied by itself for elimination of toxin contamination. But the regulatory relationship between fungal development and aflatoxin biosynthesis should also be examined for a longer term effect on the contamination of fungus as well.

POSTER PRESENTATIONS

Molecular Mechanisms Governing Aflatoxin Pathway and Gene Expression

CONSOLIDATED INFORMATION ON AFLATOXIN PATHWAY GENETICS

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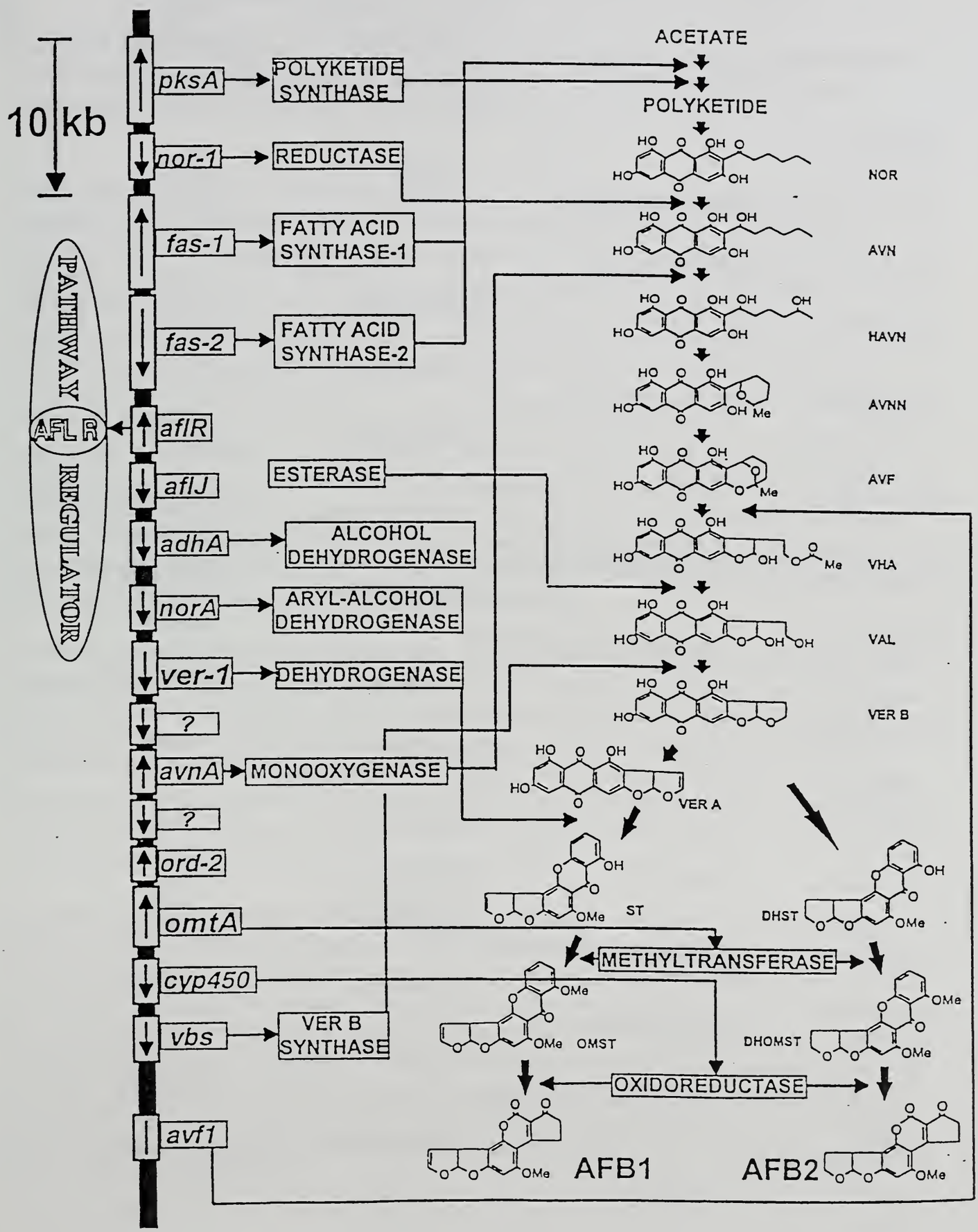
Several labs are involved in studying the molecular regulation of aflatoxin biosynthesis. A comprehensive and cooperative research effort among the scientists listed above and others such as Drs. C. A. Townsend, John Hopkins, and K. Yabe (Japan), has resulted in important breakthroughs in the last few years. The chemistry, biochemistry, and genetics of the biosynthetic pathway have been extensively characterized. The regulation of aflatoxin biosynthesis has been elucidated and the organization on the fungal chromosome of the genes involved in toxin synthesis has been understood.

A summary of the cluster of aflatoxin pathway genes, corresponding biosynthetic enzymes, and precursors involved in the aflatoxin B_1 and B_2 synthesis is presented in Figure 1. The generally accepted aflatoxin B_1 and B_2 biosynthetic pathway in *A. parasiticus* and *A. flavus,* the identified enzymes for some specific conversion steps and cloned genes are schematically presented. The regulatory gene, *aflR*, coding for the pathway regulatory factor (AFLR protein) controls the expression of the structural genes at the transcriptional level. The *fas-1, fas-2* and the *pksA* gene products, fatty acid synthase and polyketide synthase, respectively, are involved in the conversion steps between the initial acetate unit to the synthesis of the decaketide backbone in aflatoxin synthesis. The *nor-1* gene encodes a reductase for the conversion of NOR to AVN. The *avnA* gene encodes a P450 monooxygenase for the conversion of AVN to HAVN. The *adhA* (homology to an alcohol dehydrogenase), *norA* (homology to an aryl-alcohol dehydrogenase), ver-1 (encoding a dehydrogenase), *ord-2, cyp450* and *avf1* gene products have been demonstrated to be functioning at various stages of the pathway, but their exact enzymatic role has not been fully characterized and is under investigation. The *omtA* gene encodes an 0-methyltransferase for the conversion of ST to OMST and DHST to DHOMST. The *vbs* gene encodes a Ver B synthase (cyclase) which has been reported to be involved in the conversion of VHA to Ver B. The oxidoreductase and esterase have been characterized to be involved in the aflatoxin biosynthetic pathway, however, their corresponding genes have not been confirmed. The vertical bar on the left represents at least a 75 kb aflatoxin pathway gene cluster with identified genes shown in the open boxes. The names of the individual genes are labeled next to the open boxes and the un-named transcripts are labeled by question mark. Arrows inside the open boxes indicate the direction of transcription. Arrows indicate the relationships from the genes to the enzymes they encode; from the enzymes to the bioconversion steps they are involved in; and from the intermediates to products in the aflatoxin bioconversion steps. Abbreviations used in the figure: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'hydroxyaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; Ver B, versicolorin B; Ver A, versicolorin A; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-0-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2. In addition, 1-hydroxyversicolorone is an intermediate between AVF and VHA, and demethylsterigmatocystin is an intermediate between Ver A and ST.

Similar molecular genetic studies have been conducted with *A. nidulans* in the labs of Drs. N. P. Keller and T. Adams (Texas A & M University). *A. nidulans* produces the polyketide (ST) as the end-product; this metabolite is the next-to-last precursor in the aflatoxin (AF)biosynthetic pathway. The ST-gene cluster (approximately 60 kb) has been found to be located in the three overlapping cosmids on chromosome 4 in *A. nidulans.* At least 20 transcripts from this cluster, which one coregulated, have been characterized. It has also been demonstrated that both the enzymatic and regulatory gene functions between the ST gene cluster *A. nidulans* and the AF gene cluster in *A. flavus/A. parasiticus* are conserved (Figure 2).

The aflatoxin biosynthetic pathway is a common feature in aflatoxin contamination amongst all crops and has been understood in significant detail. Successful and efficient implementation of the molecular strategies for aflatoxin control have benefitted understanding of the molecular genetics of aflatoxin biosynthesis. A detailed description of the potential contributions of molecular biology to aflatoxin elimination is provided in an article published in INFORM, a popular publication of the American Oil Chemists' Society (Vol 6, No. 3, March 1995, pp 262-271).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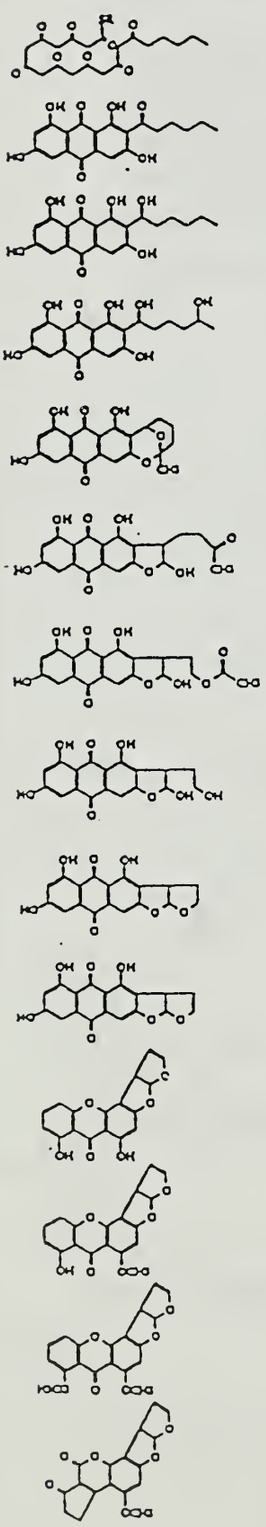
PATHWAY REGULATOR
AFLR
pksA
POLYKETIDE SYNTHASE
nor-1
REDUCTASE
fas-1
FATTY ACID SYNTHASE-1
fas-2
FATTY ACID SYNTHASE-2
aflR
aflJ
ESTERASE
adhA
ALCOHOL DEHYDROGENASE
norA
ARYL-ALCOHOL DEHYDROGENASE
ver-1
DEHYDROGENASE
?
avnA
MONOOXYGENASE
?
ord-2
omtA
cyp450
vbs
VER B SYNTHASE
avf1
ACETATE
POLYKETIDE
NOR
AVN
HAVN
AVNN
AVF
VHA
VAL
VER B
VER A
ST
DHST
METHYLTRANSFERASE
OMST
DHOMST
OXIDOREDUCTASE
AFB1
AFB2

Figure 1

Biosynthesis of Aflatoxin and Sterigmatocystin in Aspergillus spp.



	Known or Proposed Activities	Gene in A. nidulans	Gene in A. fla & A. parasitic
Acetyl CoA Malonyl CoA			
Polyketide Progenitor	FAS PKS	stcJ stcK stcA	fas-1 fas-2 pksA
Norsolorinic Acid	Dehydrogenase	stcE	nor-1
Averantin	Oxidation	stcF	avnA
5-Hydroxyaverantin	Dehydrogenase	Dehydrogenase = stcG, N	adhA
Averufin	Oxidation	Monooxygenase = stcB or W	avf1
1-Hydroxyversicolorone	BV Oxidation	Monooxygenase = stcB or W	
Versiconal Hemiacetal Acetate	Esterase	Esterase = stcI	
Versiconal	Cyclase	stcN?	vbs
Versicolorin B	Desaturation	stcL	
Versicolorin A	BV Oxidation + Oxidoreductase + Decarboxylation	stcS stcU ?	ver-1
Demethylsterigmatocystin	Methylation	stcP	
Sterigmatocystin	Methylation	Absent in A. nidulans	omtA
o-methyl Sterigmatocystin	Dioxygenation	Unknown functions: stcV, stcQ	ord-
Aflatoxin B1			

Figure 2

ANALYSIS OF THE *NOR*-1 AND *VER*-1 PROMOTER IN THE FILAMENTOUS FUNGUS *ASPERGILLUS PARASITICUS*

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Aflatoxins are highly toxic and carcinogenic secondary metabolites of certain strains of *Aspergillus parasiticus* and *A. flavus*. Aflatoxin contamination of food and feed occurs worldwide and has an enormous economic impact due to the requirement to dispose of contaminated products (3). Epidemiological data has linked aflatoxins and hepatic cancer (2). Consequently, developing the means to eliminate aflatoxin from the food chain has been a research priority for several labs across the globe. In recent years, several groups have identified and cloned several of the genes in the aflatoxin biosynthetic pathway (1,4,7,12). Perhaps the most characterized gene involved in the pathway is the *nor*-1 gene. The product of the *nor*-1 gene is responsible for converting the first stable intermediate, norsolorinic acid, to averantin near the beginning of the biosynthetic pathway (11). The *ver*-1 gene product catalyzes the conversion of versicolorin A to sterigmatocystin near the end of the biosynthetic pathway (7). The biosynthetic genes identified, including *nor*-1 and *ver*-1, all have similar timing of transcript accumulation (9) which suggests that the biosynthetic pathway genes may be coordinately regulated (6). The purpose of this study is to identify cis-acting sites in the *nor*-1 and *ver*-1 promoters responsible for their regulation.

In order to help identify cis-acting sites that may be involved in regulation of *nor*-1 and *ver*-1 expression, two different growth media were utilized. Previous work has shown that GMS (glucose plus mineral salts) is an aflatoxin inducing medium, whereas PMS (peptone plus mineral salts) is not an aflatoxin inducing medium (6). Cultures grown for 46 hours produce much less toxin, *nor*-1 transcript, and *ver*-1 transcript in PMS than in GMS (6). At 46 hours, the amount of *nor*-1 and *ver*-1 transcript and aflatoxin are rapidly increasing in GMS. Using these two growth media, nuclear protein was extracted from 46 hour cultures using a modified protocol from Nagata et al. (5) and Timberlake et al. (8).

In order to determine regions of the promoter that contain cis-acting sites, the promoters were divided into three overlapping fragments. The three overlapping fragments (R, M, and L) and the entire region (T) were amplified using the polymerase chain reaction from clones containing the appropriate regions. The amplified regions are described in table 1. For both the *nor*-1 and *ver*-1 promoter, the +1 was the transcriptional start site as determined by Trail et al. (9) and Skory et al. (7), respectively. The 5' borders of the promoters was determined by identifying the end of the neighboring upstream genes. The 5' end of nor-L is at the 3' end of the neighboring upstream gene and the 5' end of ver-L contains the 3' end of the *nor*-1 gene. Both nor-R and ver-R contain the transcriptional and translational start sites and also contain a TATA box.

Table 1: Location of the PCR amplified promoter fragments from nor-1 and ver-1

nor-L from -386 to -210	ver-L from -518 to -298
nor-M from -230 to -120	ver-M from -308 to -98
nor-R from -140 to +32	ver-R from -118 to +107
nor-T from -386 to +32	ver-T from -518 to +107

The promoter fragments were used as probes for gel shift analysis with the purified nuclear protein from both GMS and PMS. Nor-R produced a shifted complex under inducing conditions (GMS) but not under non-inducing conditions (PMS) which implicates a positive regulatory scheme and suggests that a potential critical cis-acting site is located in that region. In competition experiments, the shifted complex with nor-R can be competed away with a 250 fold molar excess of unlabelled nor-R while 250 fold molar excesses of unlabelled nor-M and nor-L compete for the nor-R shifted complex minimally. Work is underway with the *ver*-1 promoter fragments. The two promoter sequences will be compared in order to identify potential cis-acting sites that are in both promoters. In addition, DNAse I footprinting with both nor-T and ver-T is underway.

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INHIBITING THE AMYLASE PRODUCED BY *ASPERGILLUS FLAVUS* AS A STRATEGY FOR CONTROLLING AFLATOXIN PRODUCTION

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With a GUS reporter assay, an aflatoxin-inducing activity was detected previously in culture filtrates of the aflatoxigenic *A. flavus* strain NRRL 3357 grown on maize kernels. The inducing activity passed through ultrafiltration membranes with 10 KDa exclusion and was not inactivated by autoclaving for 15 minutes. A major component of the ground maize kernels was starch. Therefore, the objective of the present study was to explore the possibility that an amylase produced by *A. flavus* and the degradation products of starch have a role in the aflatoxin induction mechanism in colonized maize kernels. **This work has been accepted for publication: C. P. Woloshuk, J. R. Cavaletto and T. E. Cleveland. Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. Phytopathology.**

- The molecular data obtained in the present study confirmed much of the work published by earlier researchers and support the hypothesis that the best inducers of aflatoxin biosynthesis were carbon sources readily metabolized by glycolysis and the pentose phosphate pathway. Carbohydrates such as glucose, maltose, maltotriose, fructose and sorbitol resulted in the highest level of induction of GUS activity, whereas peptone, lactose, sorbose and glycerin were poor inducers. The glucose analogue 2-deoxy-D-glucose also was tested and found not to induce GUS activity.

- We have shown that a gene involved in aflatoxin biosynthesis was induced within 24 hr after replacement of the medium with glucose concentrations of 1mM. It was also shown that maltose and maltotriose induced GUS activity at similar concentrations. These data indicate that the mechanism regulating aflatoxin biosynthesis is activated by low levels of carbon flow and that increasing the carbon concentration accentuates the induction process.

- Analysis of the culture filtrates suggests that a single α-amylase is responsible for starch degrading activity. No evidence was found to indicate maltase or amyloglucosidase were produced to any significant level in the culture filtrates. The presence of glucose, maltose, and maltotriose in the culture filtrates suggests that these sugars are part of the aflatoxin-inducing activities.

- Increase in amylase activity in the filtrates from maize kernel cultures paralleled the increase in aflatoxin-inducing activity, suggesting that the action of amylase on the maize starch has a role in induction. Although starch is the primary component of the endosperm, the embryo also has significant amounts of starch, as much as 8% of the dry weight. We hypothesize that after embryo colonization, *A. flavus* produces an extracellular amylase that supplies a burst of fermentable sugars, and it is these sugars which induce aflatoxin biosynthesis.

- A similar pattern of amylase production was observed when *A. flavus* was grown on 0.2% amylopectin as a carbon source; however, the filtrates from these cultures did not contain measurable amounts of glucose, maltose, or maltotriose, and did not induce GUS activity. These data suggest that the products of amylopectin degradation were limiting in the culture. As a consequence, the products were likely rapidly consumed by the growing fungus and not available to stimulate aflatoxin biosynthesis. Increasing the amount of amylopectin to 2% resulted in filtrates containing aflatoxin-inducing activity and measurable amounts of sugars.

- Although the data presented are not conclusive that amylase has a role in the production of aflatoxin in infected maize, they suggest that inhibiting the action of the amylase produced by *A. flavus* may be a viable control strategy. An obvious question is: do the various plant amylase-inhibitors have any effect on amylases from *A. flavus*? Published literature suggests that many of the known amylase-inhibitors, including those in maize, have little or no activity toward fungal amylases. There is one report indicating that the inhibitor from black bean has activity against fungal amyloglucosidase, but it was not inhibitory to α-amylases. We have tested wheat (*Triticum aestivum*) amylase-inhibitor (Sigma) and determined that the inhibitor did not inhibit the amylase from *A. flavus*, but inhibited porcine pancreatic α-amylase by 90%.

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MOLECULAR AND GENETIC ANALYSIS OF A PUTATIVE AFLATOXIN BIOSYNTHESIS REGULATORY MUTANT FROM *ASPERGILLUS FLAVUS*

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K.E. Papa generated 23 non-allelic aflatoxin biosynthesis mutants in *Aspergillus flavus*. Eleven of these were mapped to linkage groups, 10 to linkage group 7, which includes genes in the aflatoxin biosynthetic cluster, and one to linkage group 2. Given the absence of a colored intermediate in the latter strain, and the physical separation of its mutation from the biosynthetic cluster, it appeared likely that the mutated gene would be of a regulatory nature. An analysis of the RNA transcripts from strain 241 (*tan, afl-4, pdx6*) indicates that the *aflR* message is absent from this strain. The lack of aflatoxin biosynthesis in transformants of strains 241 containing additional copies of *aflR*, indicate that the mutation is not due to a non-functional copy of this gene. Transformants containing an *aflR::GUS* construct failed to exhibit β-glucuronidase activity, indicating that the *aflR* promoter is not active in this strain. Transformants provided with *aflR* message, due to the presence of a construct expressing *aflR* via a heterologous promoter, were restored in both *aflR* transcript and aflatoxin biosynthesis. These data allow us to putatively identify the *afl-4* mutation as an upstream regulatory gene controlling the transcriptional activity of *aflR*. Strain 241 exhibits no abnormalities in development or morphology, thus distinguishing it from developmental mutants which have been shown to also be affected in aflatoxin biosynthesis. Therefore, the gene product of *afl-4* may be more likely to provide a target for control of aflatoxin biosynthesis than mutants which affect both development and secondary metabolism.

DELETION ANALYSIS OF THE *ASPERGILLUS FLAVUS aflR* Promoter

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The *Aspergillus flavus aflR* gene, a pathway specific regulatory gene, regulates the aflatoxin biosynthetic pathway. Transcription of the *aflR* is also regulated. The gene is contiguous to, but divergently transcribed from the *aflJ* gene in the aflatoxin gene cluster. The untranslated region between the two transcriptional start points (tsp) is 542 bp long. The elements in the 5' untranslated region (UTR) of *alfR* required for expression and regulation of *alfR* are not known. To study the response elements in the 5' UTR of *aflR*, we fused the 737 bp intergenic region between *aflR* and *aflJ* to the β-glucuronidase reporter gene. Here we present the initial characterization of the 5' UTR of *aflR*. The results of deletion analysis indicate that a 5' UTR of more than 113 bp is required for transcriptional activity and that constructs with 5' UTR greater than 415 bp respond similarly to a construct with a 737 bp UTR. We found that the deletion of a 19 bp region from the 415 bp construct resulted in GUS expression 10-20 fold higher than the 737 bp construct. A deletion construct lacking the 19 bp region retained the capacity to be induced on media conducive to aflatoxin production. The data suggest that there are at least two regions within the 415 UTR of *aflR* that modulate transcription of *aflR*. A 19 bp region appears to be involved in the level of transcription and another region not yet described appears to be involved in induced expression of *aflR*.

OVERPRODUCTION OF AFLATOXIN PATHWAY INTERMEDIATES AFFECTS SCLEROTIAL DEVELOPMENT AND PRODUCTION IN *ASPERGILLUS PARASITICUS*

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Aflatoxin biosynthesis has been repeatedly suggested to be associated with sclerotial production in *A. parasiticus* and *A. flavus*. Sclerotia are dense, compacted aggregates of mycelia. They are multicellular resting survival structures that are resistant to unfavorable conditions and capable of remaining dormant for long periods of time. Physical and environmental factors, such as light, temperature, pH, C/N balance, nutritional requirements and inhibitors on sclerotial production have been studied (1,2). Certain isolates produce aflatoxin but not sclerotia and vice versus (2). Nonetheless, in *A. flavus* strains that do produce aflatoxin and sclerotia, aflatoxin production and sclerotial formation seem to be inversely related to pH change of the agar medium, i.e., decrease in pH stimulates aflatoxin production but inhibits sclerotial formation. Furthermore, sclerotial maturation seems to be coincident with the cessation of aflatoxin production in *A. flavus* (3). Although no correlation exists between the level of sclerotia production and aflatoxin accumulation, *A. flavus* strains producing higher aflatoxin tend to have smaller sclerotia (4). Accumulation of versicolorin A has been implicated in the inhibition of sclerotial production in *A. parasiticus* ATCC 36537 (6). On the contrary, the accumulation of an early aflatoxin pathway intermediate, norsolorinic acid, in *A. parasiticus* ATCC 24690 does not affect sclerotial production (7). However, elimination of the accumulation of any aflatoxin intermediates enhances the level of sclerotial production (5,7). These observations indicate that aflatoxin biosynthesis and sclerotial development are related cellular processes.

A genetic relationship between aflatoxin biosynthesis and sclerotial development was examined in *A. parasiticus* SRRC 2043. This strain, which accumulates 0-methylsterigmatocystin, demonstrated an overexpression of the aflatoxin pathway genes, *aflR* and *aflJ*, when transformed with additional copies of these genes (*aflR* is a pathway regulatory gene and *aflJ* has been demonstrated by Payne and coworkers to affect aflatoxin production). Elevated levels of aflatoxin intermediates were produced by introduction of the extra copies of either *aflR* or *aflR* plus *aflJ* in transformants, but not by the transformed *"aflJ"* alone. The number of sclerotia produced in *aflR* and in *aflR* plus *aflJ* transformants on PDA plates increased significantly, but no change was observed in the *aflJ* transformant. This increase in the number of sclerotia was concomitant with a decrease in the sclerotial size. However, the sclerotial number of the *aflR* plus *aflJ* was substantially decreased on CZ plates. An increase in the production of aflatoxin intermediates resulted in a change in sclerotial morphology as well; the regular round/oval shape was modified to elongated/bullet shape depending on the medium used. Scanning electron micrographs showed that the sclerotia of the *aflR* plus *aflJ* transformant was not as compact as that observed for the wild-type strain (Figure). These results suggest a regulatory association between sclerotial morphogenesis, aflatoxin biosynthesis, and possibly other cellular processes.

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A. parasiticus SRRC 2043 Overproducing transformant

20X

50X

ISOLATION AND CHARACTERIZATION OF AN EXPERIMENTALLY-INDUCED, AVERANTIN-ACCUMULATING MUTANT OF *ASPERGILLUS PARASITICUS*

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Transformation of *A. parasiticus* RHN1 with a *norA* disruption construct fortuitously gave rise to a transformant that was found to have undergone an approximate 6 kb deletion of a region of the aflatoxin biosynthetic pathway. The deletion event resulted in the partial and complete loss of the known aflatoxin biosynthetic genes *avnA* and *ver-1*, respectively. In addition, a significant portion of the *norA* dehydrogenase gene was deleted though it's exact role in aflatoxin biosynthesis has not been determined. Another putative aflatoxin biosynthetic gene that is located proximal to the 3' end of *ver-1* was also completely deleted. It's function has not been determined at this time but it demonstrates homology with P450-monooxygenases. Due to the loss of function of the *avnA* gene in the deletion mutant, it was unable to further metabolize averantin, and therefore this metabolite was the main polyketide found in the product mixture. However, besides averantin, methylaverantin, represented 16% of the recovered product mixture. Previous studies of phenolic metabolites produced by the Aspergilli found that *Aspergillus versicolor* accumulated methylaverantin as one of its main polyketide metabolites. To our knowledge, this compound has not been previously reported in cultures of *A. parasiticus* or *A. flavus*. One area of intense interest has been in the development of biological control agents to reduce levels of aflatoxin contamination in field crops. The averatin-accumulating mutant described in this report should make for an excellent biocontrol agent with respect to control of aflatoxin contamination of peanut. Unlike the strains currently being utilized as biocompetitive agents, the exact mutational event that resulted in loss of aflatoxin biosynthesis in the deletion mutant has been well characterized. In addition, the readily observable reddish-orange pigmentation (due to averantin accumulation) of the deletion mutant make it desirable for ease of quantitation of fungal spread and population levels in the field. Also, averantin is an early intermediate in aflatoxin biosynthesis and does not contain the dihydrobisfuran moiety that has been implicated in aflatoxin's carcinogenicity upon ingestion.

PLATFORM PRESENTATIONS

Potential Use of Native Plant Compounds for the Prevention of Aflatoxin Contamination

A CORN METABOLITE THAT INHIBITS AFLATOXIN SYNTHESIS

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(Collaborators: D. Bhatnager, F. S. Chu, and D. G. White)

Research in my lab is focused in two areas: the regulation of aflatoxin biosynthesis; and the identification of compounds in corn seeds inhibitory to aflatoxin accumulation. A brief description of each area is discussed below.

Last year I reported the initial characterization of a compound from corn inbred Tex6 that inhibits aflatoxin formation. Further studies on extracts from Tex6 have shown that there are two inhibitory compounds in these seeds, one compound inhibits growth of *A. flavus* and thus aflatoxin accumulation, and the other compound inhibits aflatoxin biosynthesis but not fungal growth. Both of these compounds are proteinaceous. Based on SDS-PAGE, we estimate the molecular weight of the growth inhibitor to around 25,000 to 50,000 daltons. This compound causes a 50% reduction in growth at 35 ug/ml. The compound inhibiting aflatoxin biosynthesis appears to have a molecular weight greater than 100,000 daltons. This compound causes a 50% inhibition aflatoxin biosynthesis at 75 ug/ml. Because these compounds are proteinaceous, that can be used in two strategies to inhibit aflatoxin accumulation. Our first strategy is to prepare antibodies to these compounds and use the antibodies a chemical markers to follow the segregation of resistance in breeding populations. These chemical markers will be associated with genetic markers on an RFLP map of corn being developed by T. R. Rocheford and D. G. White. A goal of the research is to associate chemical markers with specific genes for resistance. The ability to associate the presence of compounds conferring resistance to aflatoxin accumulation with genetic markers will allow marker assisted selection to follow the movement of resistance into commercially important lines. A second strategy for the use of these compounds to control aflatoxin accumulation is to clone the plant genes coding for these products, and to use these genes to develop transgenic resistance in corn, cotton, peanuts and tree nuts.

A second research area in the lab is focused on understanding the regulation of aflatoxin biosynthesis. We have characterized a gene, *aflJ*, that resides within the aflatoxin biosynthetic cluster and whose profile of transcription follows that of aflatoxin biosynthesis. The gene is located adjacent to the pathway regulatory gene, *aflR*, and the two are divergently transcribed. The location of *aflJ* in relation to *aflR* and its profile of transcript accumulation suggested to us that this gene may be involved in aflatoxin biosynthesis, and possibly in the regulation of aflatoxin biosynthesis. We showed by gene disruption that *aflJ* is required for aflatoxin biosynthesis. To determine the possible function of *aflJ* in aflatoxin biosynthesis, metabolite conversions studies were done. Feeding the disruptant with the pathway intermediates norsolorinic acid, sterigmatocystin, or o-methyl sterigmatocystin did not result in the accumulation of aflatoxin. Further, no colored or fluorescent pathway intermediates were observed in extracts of the disrupted strain. Thus, it appears that enzymatic activities necessary to convert the pathway intermediates are not active in the strain with a disrupted copy of *aflJ*. These data suggested that *aflJ* may be involved in the transcriptional regulation of the

pathway. Northern analysis of the disrupted strain showed that no transcripts were present for *aflJ*, but transcripts were present for *pksA*, *nor1*, and *omt1*. Thus it does not appear that *aflJ* is involved in the transcriptional regulation of aflatoxin biosynthesis. Sequence analysis of the gene and the putative peptide revealed no homology to known genes or peptides in the database and no enzymatic motifs. The putative peptide does have three regions with predicted homology to membrane spanning domains. The only other motif in the putative peptide encoded by the gene is a perfect AAA signature. Proteins with this signature are associated with many cellular activities including protein transport and peroxisome biogenesis. All of the known AAA proteins, however, contain an ATP binding site not present in *aflJ*. At this point we do not know if *aflJ* is involved in any of these functions. It does appear that *aflJ* plays a key role in aflatoxin biosynthesis and because it prevents the accumulation of pathway intermediates, it is a good target for inhibition of aflatoxin biosynthesis.

USE OF NATURAL PRODUCTS TO CONTROL INFECTION AND GROWTH OF AFLATOXIGENIC ASPERGILLI IN TREE NUTS

Plant Protection Research Unit, USDA, ARS, Western Regional Research Center, Albany, CA (presented for the unit by Bruce Campbell)

The Plant Protection Research Unit is a new participant in the Aflatoxin Elimination Workshops. The unit includes a multidisciplinary team of chemists, microbiologists and entomologists. Members of this unit are currently identifying natural products and microbial agents that reduce or eliminate aflatoxins in certain tree nuts namely, pistachio, walnut and almond. The three basic goals of this research are to isolate and identify natural products or microbial agents which: 1) disrupt aflatoxin biosynthesis, 2) are antibiotic towards aflatoxigenic aspergilli, and 3) control insect pests of tree nuts by disrupting normal growth, feeding, reproductive or host-finding behaviors. This third goal stems from the fact that wounds in tree nuts from feeding by certain insects are a major avenue of infection by aflatoxigenic aspergilli.

The team devoted to identifying natural resistance factors in tree nuts which confer resistance to aspergilli or suppresses biosynthesis of aflatoxin includes chemists Noreen Mahoney and Russell Molyneux. Using *in vitro* experiments, they find vastly different levels of aflatoxin are produced in kernels of different commercial nut varieties inoculated with *Aspergillus flavus*. This variability is indicative of regulation of aflatoxin biosynthesis by natural constituents in the host-plant which could be genetically manipulated. Initial attempts to identify such factors have focused on pistachios. Extracts of pistachio hulls, seedcoats and kernels were made by sequential extraction with non-polar and polar solvents. The highest level of aflatoxin suppressant activity is found in the ethyl acetate and aqueous fractions. Initial results indicate the active components are phenolic compounds and hydrolyzable tannins.

In addition to searches for natural products of nuts to control aflatoxin, microbiologists Nelson Goodman and the team of Jim Baker, Ok-Koo. Grosjean, lead by Sylvia Hua, are examining natural microbial biocontrol agents and their natural products which reduce or eliminate aflatoxins in tree nuts. The 'Hua' team devised a bioassay using a mutant form, *nor*, of *Aspergillus* which does not complete biosynthesis of aflatoxin but, instead, accumulates norsolorinic acid, an intermediate in the biosynthetic pathway of aflatoxin. This compound is visible as a bright orange pigment in this mutant strain. Potential yeast biocontrol agents are scored for inhibitory activity in spore germination, colony expansion and sporulation of *nor* mutants. This visual assay is a safe approach to effectively screen for yeast strains that suppress the growth of aflatoxigenic aspergilli. The phenolics, acetosyringone, syringaldehyde and sinapinic acid, were found to prevent biosynthesis of aflatoxin. When tested on the *nor* mutants, they prevented synthesis of norsolorinic acid, thus indicating they have an inhibitory effect in the early steps of the aflatoxin biosynthetic pathway.

The effort to prevent insect feeding damage to tree nuts includes entomologists Doug Light, Nathan Schiff, Chris Mehelis and Bruce Campbell, and chemist Gloria Merrill. The goal of this team is to develop new, effective and environmentally sound control strategies against moths that attack tree nuts. One novel approach to controlling these insects uses semiochemicals, natural volatile chemicals which influence insect behavior. Such compounds can either

emanate from the host plant or from the insects. Sex pheromones are currently used in orchards to monitor moth populations. The chief control strategy using semiochemicals is to permeate orchards with synthetic sex pheromones, thereby disrupting moth mating. We discovered that addition of certain host-plant volatiles (HPVs) to moth sex pheromones enhances attractancy up to almost three-fold. This level of synergistic enhancement by HPVs should enable sex pheromones to be effective mating disruptants in orchard environments. To date, HPVs discovered include methyl octanoate, a peach volatile effective against peach twig borer (PTB) in almonds, and blends of walnut monoterpenes or apple and pear alcohols or esters for codling moth (CM) in walnuts. In addition, gas chromatographic/ mass spectroscopic searches are used to identify chemical volatiles emitted by nuts. We are focusing on those volatiles emitted at hull-split in almonds which attract navel orangeworm (NOW). We are also examining host-race formation in these moth pests. These moth species have a wide host-plant range which could effect efficacy of sex pheromone/HPV based control strategies. A wide geographic and host-plant range of these pests were sampled and a search is underway for polymorphic mitochondrial DNA (mtDNA) restriction fragment length polymorphic markers (RFLPs). RFLPs, determined by probing Southern blots with probes generated by PCR amplifying the entire mtDNA genome of respective moth species, will be used to identify uniquely isolated moth populations. In addition to these seriochemical/ population studies, we are also incorporating natural products, isolated by the chemical team, into artificial diets to find compounds which disrupt insect feeding and development.

AFLATOXIN INHIBITORS AND STIMULATORS FROM OILSEEDS

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Oilseeds contain components which interact with *Aspergillus flavus* to either inhibit or stimulate aflatoxin production. Seedcoat tissue of developing cottonseed contains factors inhibitory to aflatoxin biosynthesis in axenic cultures. The inhibitory activity is nondialyzable, heat stable, pronase resistant, periodate-sensitive and concentration dependent. The major aflatoxin-inhibitory fraction did not bind to either anion exchange or cation exchange media and, thus, was neutral in charge. The cotton seedcoat inhibitor (CSCI) material was purified on a BioGel P-100 column eluted with a Tris/NaCl buffer (pH 9.0). The purest CSCI material was obtained from the void volume (V_O) peak. The major component of this fraction was a xylan (> 90 % xylose). Aflatoxin production was inversely related to CSCI concentration in fermentation medium (log AFB_1 *vs* log [CSCI], r^2=0.82, P<0.002). The ED_{50} of the crude CSCI material was 173 μg per mL, whereas the ED_{50} of the V_o CSCI was 6.2 μg per mL. Comparison of the ED_{50} values indicates a 28-fold purification. These data support the hypothesis that the cotton seedcoat inhibitor of aflatoxin biosynthesis is associated with a seedcoat-specific xylan. Fungal hydrolytic enzymes may digest CSCI into smaller molecules which are also active. It is possible the active component is not a carbohydrate, but, instead, another bioactive molecule (e.g., peptide) covalently linked to the xylan. The inhibitor moiety may bind to the fungal cell wall in order to initiate its effects. An improved understanding of the mechanism through which CSCI acts may lead to insights on strategies to interfere with the contamination process. Other oilseed components cause stimulation of aflatoxin production. Since seed-specific storage proteins comprise a significant proportion of seed dry weight in both corn and cotton, an investigation was undertaken to determine effects of these proteins on fungal growth and mycotoxin production. In a defined medium containing sucrose and NaNO3, supplementation with bovine serum albumin (BSA), collagen, cottonseed storage protein (CSP) or zein induced elevated aflatoxin levels in axenic cultures of *A. flavus*. In medium using protein as the sole carbon/nitrogen source, low levels of aflatoxin were produced with BSA, CSP, or zein. Only collagen produced aflatoxin levels comparable to defined medium controls. A dose response study using CSP as the sole carbon/nitrogen source revealed that aflatoxin (r^2 =0.96, P<0.05) and biomass (r^2=0.99, P < 0.05) production were correlated with protein concentration. Zein similarly could serve as both a sole nitrogen and sole carbon source. In addition, zein-containing medium with sucrose, but without nitrate, produced aflatoxin levels up to 8 times greater than the standard aflatoxin production medium. Both CSP and zein induced production and secretion of a 35 kDa metalloprotease in *A. flavus* cultures, when no defined carbohydrate was present in the medium. The results suggest that seed storage protein composition may quantitatively influence aflatoxin contamination in oilseed crops. Micromanipulation of storage proteins may reduce aflatoxin susceptibility of oilseed crops, while retaining nutritional properties.

STRUCTURE/ACTIVITY STUDIES OF SOME INHIBITORS OF *A. FLAVUS* AFLATOXIN B_1 SYNTHESIS WHICH OCCUR IN CORN KERNELS

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The objective of this work was to evaluate selected metabolites of corn and related compounds for their effect on growth and aflatoxin B_1 (AFT B_1) production by *Aspergillus flavus* NRRL 3357 and determine how representative NRRL 3357 is as a strain for testing. The suspended disc culture method (RA Norton, 1995, Mycopath. 1 29:103-109) was used in evaluating the compounds discussed.

The anthocyanin aglycones pelargonidin, cyanidin, peonidin, delphinidin, luteolinidin, apigeninidin, and malvidin were tested at concentrations from 0.3 - 9.7 mM (ca. 0.1 - 3.0 mg/mL). Compounds lacking a hydroxy group at the 3-position of the heterocyclic ring, luteolinidin and apigeninidin, were significantly less inhibitory than the corresponding 3-hydroxy compounds. Mono and diglycosyl forms of pelargonidin and cyanidin were tested to determine if glycosylation would affect the ability of these compounds to inhibit toxin formation. The monogylcosides were ca. 25% less inhibitory than the aglycones. Additional flavonoid compounds with 3-hydroxy, or 3,4-dihydroxy B-rings and different oxygenation patterns on the heterocyclic ring were tested to determine if non-charged flavonoids might be active. Kaempferol, naringinen, luteolin, quercetin, taxifolin and catechin were tested. Of these compounds only (+)-catechin, luteolin and kaempferol showed significant inhibition. A double bond at the 2-position was essential for activity.

Carotenoids were tested at levels from 0.032 to 1000 μg/mL for C_{40} compounds and equimolar levels for others. Highest inhibition correlated with the presence of an α-onone type ring structure in lutein and α-carotene. Comparison of activity with α-, and β-ionone showed strong inhibition by α-ionone and less effect from β-ionone, comparable to the difference shown by α/β-carotene and lutein/zeaxanthin. β-Cryptoxanthin (4-hydroxy-β-carotene) also showed strong inhibitory activity as did lycopene at higher concentrations. Canthaxanthin was a weak inhibitor due to β-ionone rings with keto groups at the 4,4 positions. The carotentoid derivatives, retinol, retinal, retinoic acid, retinol acetate and 13-cis-retinol were tested at levels equimolar with C_{40} carotenoids. Inhibition was greatest for retinol acetate (I_{50} 0.53 mM, concentration for 50% inhibition) and least for retinal (I_{50} 0.6 mM). Retinoic acid was least inhibitory at the higher concentration.

To determine the point in the aflatoxin biosynthetic pathway at which the carotenoids were acting, α-carotene, kaempferol and delphinidin were incubated with an *A. parasiticus* mutant (SRRC 164) which accumulates norsolorinic acid. Kaempferol stimulated toxin levels of SRRC 162, contrary to results with NRRL 3357. The results for delphinidin and α-carotene showed no accumulation of intermediates and pronounced inhibition of AFB_1 and norsolorinic acid (10% and 2% of control, respectively) by delphinidin and α-Carotene (22%-90% and 8%-16%). Indicating that inhibition occurs early in the pathway for these two compounds, possibly the polyketide synthase.

Four of the natural corn aflatoxin B_1 inhibitors found in the work described above were tested pairwise to determine if interference in inhibition would occur. All aflatoxin levels for the

combinations were within experimental error for the product of the separate inhibition values; e.g., β-carotene and pelargonidin produced 40% and 67% inhibition individually and 82% together. The results indicated that combinations of these compounds do not interfere with each other.

Additional *A. flavus* and *A. parasiticus* strains were tested against β-carotene (50 μg/mL) to determine how representative NRRL 3357 is in its response. *A. flavus* strains had from 89% to 96% inhibition and were significantly more sensitive than NRRL 3357 which averages ca. 50% inhibition. *A. parasiticus* strains were less sensitive and generally similar in inhibition to NRRL 3357. This and other data strongly indicate that NRRL 3357 is a conservative indicator of aflatoxin inhibition.

POSTER PRESENTATIONS

Potential Use of Native Plant Compounds for the Prevention of Aflatoxin Contamination

HOST-PLANT VOLATILES AS PUTATIVE SEX PHEROMONE SYNERGISTS OF MOTH PESTS ASSOCIATED WITH *ASPERGILLUS* INFECTION OF TREE NUTS

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The goal of our research project is the development of new, effective and environmentally sound control strategies against moths that attack tree nuts and foster *Aspergillus* infections. Because insect feeding damage provides the avenue for *Aspergillus* invasion and infection of tree nuts, then fundamental to the elimination/reduction of aflatoxin is the development of effective control and pest management practices against moth pests. These novel pest controls will utilize and manipulate "semiochemicals" or natural volatile compounds from both host-plants and insects that evoke insect behaviors (e.g., attraction and oviposition). The chief semiochemical strategy registered for control of moth pests is "mating-disruption," in which moth sex pheromones permeate entire orchards, thereby confusing/disrupting male moth attraction and mating. However, presently this pheromone-based mating disruption technique needs improvement in its efficacy to be an effective control competitive with conventional insecticides. The key to improving the efficacy of pheromone-based semiochemicals for moth control is the discovery and development of greater attractant potency while maintaining target specificity.

Our research hypothesis is that volatile odorants identified from non-nut, alternative hostplants (e.g., peaches, stonefruits, apple, pear, etc.) will modify the attractancy of commercial formulations of pheromones of moths nut-pests when presented in a nut orchard context. Female moths perform all their sexual - reproductive activities (pheromone "calling," courtship, mating and oviposition) while perched on their host-plants. Thus, pheromones and host-plant volatiles (HPVs) always share the same environmental context and might be adaptively associated in chemoreception and behavior of responding male moths. Thereby, our research objective is to discover HPVs that enhance or synergize sex-attractant pheromones, by means of increasing the pheromones' inherent attractancy, specificity, and effectiveness in monitoring and mating-disruption of nut pest populations.

Our experimental approach is to: 1) collect and identify (GC-MS) headspace odor emissions of host-nut trees and alternative host fruit trees , 2) pick candidate compounds by comparing common vs. novel volatile constituents of host-plants, and 3) field bioassay host-plant derived volatiles for: their inherent attractancy alone, and their synergism or disruption when coevaporated with commercial pheromone. A dual-choice design using paired-pheromone traps was used to assess both attraction and preference of male moths. Pheromone traps were hung in orchard trees as pairs (in close competitive proximity): a pheromone only baited-trap vs. a pheromone + HPV baited-trap. Tests were conducted in almond orchards (for peach twig borer) and walnut orchards (for codling moth).

We discovered that the coevaporation of certain HPVs with the moth sex pheromones will increase/enhance by 74% to 150+% the pheromones' attractancy to and preference by male moths. The effective HPV synergists we've discovered are: 1) methyl octanoate (a peach volatile) for the peach twig borer (in almonds) and 2) blends of walnut monoterpenes or apple/pear alcohols or esters for the codling moth (in walnuts). This synergistic enhancement by HPVs should enable sex pheromones to be more effective in control strategies for these tree nut pests.

HOST-PLANT VOLATILES ENHANCE THE SEX PHEROMONE ATTRACTION OF THE CORN EARWORM, A KEY CORN AND COTTON PEST ASSOCIATED WITH *ASPERGILLUS* INFECTION

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Helicoverpa zea, is the principal insect pest of a wide range of valuable commodities, and thus has earned many common names: "Corn Earworm," "Cotton Bollworm," "Tomato Fruitworm," and "Soybean Podworm." *H. zea* causes losses (crop damage and required control measures) that approach one billion dollars annually in the USA. *H. zea* larvae bore into fruits of their host, thereby allowing *Aspergilli* and other economically damaging molds to enter corn ears and cotton bolls. Therefore, fundamental to the elimination/reduction of aflatoxin is the development of effective control and pest management practices against insect pests that create invasion avenues for *Aspergillus* infection.

The goal of prior research was to develop biorational, non-insecticidal, means to control *H. zea* through use of insect behavior modifying semiochemicals; both insect-produced sex-attractant pheromones and host-plant volatiles (HPV) or kairomones. A key limitation on the use of semiochemicals for insect control is that current pheromone-based monitoring and control systems are in need of greater attractant potency while maintaining target specificity. Our research hypothesis is that volatile odorants found in common among a number of host-plants will modify the attractancy of commercial formulations of the *H. zea* pheromone. The rationale is that 1) female moths "call"/release-pheromone, court, mate, and oviposit while perched on their host-plants, 2) HPVs evaporate simultaneously with pheromone emissions (i.e., share the same environmental context), and 3) thus, they might be adaptively associated in the chemoreception and behavior of responding male moths. Therefore, our research objective is to discover HPVs to enhance or synergize sex-attractant pheromones to increase attractancy, specificity, and effectiveness of monitoring and mating-disruption of *H. zea* populations.

Our experimental approach is to: 1) collect and identify (GC-MS) headspace odor emissions of host-plants; 2) pick candidate compounds by comparing common vs. novel volatile constituents of host-plants; and 3) field bioassay host-plant derived volatiles for: their inherent attractancy alone, their synergism or disruption when coevaporated with commercial pheromone, and their ability to lower the pheromones behavioral threshold. For most tests, paired-pheromone traps were used to assess both attraction and preference of male moths. Pheromone traps were erected in corn fields as pairs (in close competitive proximity): a pheromone only baited-trap *vs.* a pheromone + HPV baited-trap. Each trap was baited with a commercial pheromone-impregnated rubber septum (3 mg/septum, Trécé Inc. Salinas, CA). The individual plant volatiles evaporated from either small capillary tubes [e.g., (Z)-3-hexenyl acetate] or impregnated rubber septa.

We found that 1) HPVs had no inherent attractancy alone; 2) capture of male moths in pheromone traps was significantly increased by 18 HPVs, the best synergist being (Z)-hexenyl acetate; 3) HPV-synergists competitively lower the concentration threshold for pheromone attraction; and 4) HPV synergists did not alter the pheromone's sex or species specificity, with only males and conspecifics attracted. The discovery of HPV-synergism bolsters optimism for the future development of more highly potent and specific pheromone monitoring and control systems.

TWO INHIBITORY PROTEINS FROM CORN SEEDS, ONE INHIBITING *A. FLAVUS* AND ONE INHIBITING AFLATOXIN BIOSYNTHESIS

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Field studies have identified inbred lines of corn with resistance to aflatoxin accumulation. Genetic analyses of these inbreds show that there are two types of resistance to aflatoxin accumulation. One type of resistance appears to be directed at fungal growth, whereas the other type of resistance is to aflatoxin accumulation and has little effect on fungal growth. We report in this poster the characterization of extracts of the resistant corn inbred Tex6, and show that this line possesses at least two proteaceous inhibitory compounds. One compound inhibits fungal growth, and consequently aflatoxin accumulation, and the other compound has little effect on fungal growth, but inhibits aflatoxin formation. The ED50 values for the partially purified growth inhibitor and aflatoxin biosynthesis inhibitor are 60 and 75 ug/ml, respectively. Based on SDS-PAGE, we estimate the molecular weight of the growth inhibitor to be about 25,000-50,000 daltons. Less is known about the aflatoxin inhibitor, but its molecular weight appears to be greater than 100,000 daltons. Because both of these compounds are proteaceous, they can be used in two strategies for developing resistant corn. First, antibodies will be prepared to the inhibitors and these antibodies will be used to follow the segregation of the inhibitors in breeding populations. This will allow for a more rapid movement of these compounds into high yielding commercial lines. Second, we plan to clone the genes coding for these compounds and use them to develop transgenic plants resistant to aflatoxin accumulation.

EFFECTS OF PLANT LIPOXYGENASES ON MYCOTOXIN PRODUCTION AND DEVELOPMENT IN *ASPERGILLUS* SPP.

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(* Equal contribution to Poster)

INTRODUCTION

The carcinogenic compounds aflatoxin (AF) and sterigmatocystin (ST) are products of the same secondary metabolic pathway and are found in several fungal genera, most notably the genus *Aspergillus*. In addition to the serious health risk these compounds pose, continual AF/ST contamination of a vast array of agricultural products has seriously impacted agricultural and economic policies worldwide. The research on the biosynthesis and regulation of these toxic secondary metabolites in fungi, together with studies on the interaction between plants and fungi, could provide basic understanding that would lead to the design of novel targeted control strategies. In this regard, the biotechnological manipulation of plant compounds with antifungal properties is currently gaining relevance as a means to eliminate plant diseases. Recently the plant metabolite 13-hydroperoxy linoleic acid 13(S)-HPODE, produced by soybean lipoxygenase 1 (Gardner 1995), was shown to reduce or inhibit the biosynthesis of these carcinogenic fungal compounds (Burow *et al.*, 1996). Our interest is focused on the study of the mechanism(s) through which 13(S)HPODE inhibits the AF/ST biosynthetic pathway.

Because 13(S)-HPODE affects fungal growth as well as AF/ST production (Burow *et al.*, 1996), we hypothesize that 13(S)-HPODE AF/ST effects are a consequence of perturbations on fungal development. Interestingly, fungal hydroxylinoleic acids known as psi factors--similar in structure to 13(S)-HPODE and other plant hydroperoxides--have been isolated from *A. nidulans* mycelium. Psi factors appear to act as fungal pheromones and, accordingly, effect sexual sporulation in *A. nidulans* (Champe, 1991). To investigate a possible pheromone effect of 13(S)-HPODE on fungal development, we have transformed the ST producing fungus *A. nidulans* with the soybean lipoxygenasel gene, LOX1. Our results indicated that LOX1 expressing A. *nidulans* strains are reduced in their ability to reproduce sexually. This fact could indicate a possible connection between AF/ST production and [sexual*] development.

* whereas only *A. nidulans* has a sexual stage (where ascospores are formed in a cleistothecium), it is possible that *A. parasiticus* and *A. flavus* respond to the same pheromone in a developmentally regulated way. For example, it has often been postulated that sclerotial formation in *A. parasiticus* and *A. flavus* corresponds to cleistothecial formation in *A. nidulans*.

MATERIALS AND METHODS

Strain and maintenance

Aspergillus nidulans FGSC237 (*yA2*, *paba*A1, *trp*C801 *ve*A1) was stored as a silica stock. Fresh cultures were grown at 37 C on minimum medium (MM) (Cove, 1966) supplemented with *p*-aminobenzoic acid and tryptophan.

Construction and isolation of plasmids

Two plasmids (pCN3 and pCN4) were constructed with the soybean lipoxygenase (*lox1*) gene attached to two characterized inducible promoters, *niiA* and *alcA*, respectively (Figure 1A and 1B). pCN3 contains the *niiA(p)::lox1* fusion which is inducible by nitrate and suppressed by ammonium. pCN4 contains the *alcA(p)::lox1* fusion which is inducible by threonine and ethanol and suppressed by high concentrations of glucose. Both plasmids also contain a 1/2 *trp*C which can target them to the *trp*C locus in *A. nidulans*. A third plasmid, pTAll, containing a 4.4 Kb X*ho*I-fragment of the *trp*C gene was used as a control.

Transformation of *A. nidulans* with soybean *lox1*

A. nidulans was transformed with pCN3 or pCN4 alone or cotransformed with pCN3 + pTAll or pCN4 + pTAll using standard procedures. Transformation with pTAll alone was the control.

DNA of all tryptophan auxotrophs was probed with radiolabeled loxl for detection of *lox1* in *A. nidulans* genomic DNA.

Northern analysis of *A. nidulans loxl* transformant strains

To induce the *alcA(p)::lox1* construct, cultures were first grown in liquid glucose MM and then switched to liquid threonine MM. Mycelia were collected 0, 2, 4 and 12 hr after the switch and extracted for total RNA which was probed with *alca* and *lox1*.

Ascopore Development

Selected transformants and wild type controls were grown at 37°C on different solid media (SM):

Experiment SM #1: MM and MM plus 1% peanut oil.

Experiment SM #2: MM with nitrate (sodium nitrate) as nitrogen source and MM with ammonium (ammonium tartrate) as nitrogen source. The former medium induces *niiA(p)::loxl* expression and the latter represses *niiA(p)::loxl* expression.

Experiment SM #3: MM with l0g/L of glucose as carbon source and MM with increased glucose, up to 30 g/L. Increasing amounts of glucose repress *alcA(p)::lox1* expression.

Experiment SM #4 and #5: MM plus gossypol (a potential lipoxygenase inhibitor; Bisakowski *et al.*, 1995) at different concentrations (4 μM, 40 μM and 400 μM). The solvent utilized to dissolve gossypol was acetone (4 ml per liter of MM).

Fungi were grown on the above medium for 5, 10 and/or 22 days thereupon a 7mm plug was cored from the medium and vortexed in 400 μl Tween 20 water for 30" to release ascospores from cleistothecia (ascospores are the sexual spores of *A. nidulans* and are produced in a fruiting body known as a cleistothecium). The samples were then analyzed with a hemacytometer for total ascospore number.

Statistical analysis

Experiments were replicated and analyzed using Systat software.

Thin layer chromatography (TLC) analysis

Cores from selected 7mm plugs were extracted for ST content after counting total ascopores. Equal volumes of acetone and chloroform were added to the ascospore mixture and organic phase was extracted after thorough vortexing. Extracts were dried down under a fume hood, resuspended in 20 μl chloroform and then spotted on a precoated TLC plate (Analtech, Inc.) along with 100 ng of sterigmatocystin (ST) standard (Sigma). Samples were resolved using toluene-ethyl acetate-glacial acetic acid (80:10:10 v/v/v). The plates were then dried, sprayed with 20% aluminum chloride in 95% ethanol, and baked at 95°C for 10 min.

RESULTS

A. nidulans loxl transformants. Cotransformations with pCN3 + pTAll or pCN4 + pTAll yielded numerous transformants, many containing the *lox1* gene incorporated randomly in the fungal genome. Transformation with pCN3 or pCN4 alone yielded one transformant each (TCN3.11 and TCN4.1 respectively) where a single copy of the *loxl* was inserted in the *trp*C locus. Transformation with pTAll also yielded several tryptophan auxotrophs (containing no *lox1)*. The following transformants were used for our studies:

TCN3(niiA(p)::lox1): TCN3.4, TCN3.5, TCN3.7, TCN3.8
TCN4(alcA(p)::lox1): TCN4.1, TCN4.4, TCN4.7, TCN4.8
Controls (transformant with only pTAll incorporated): TTA11.1, TTA11.3, TTA11.4,, TTA11.5

Lox1 Expression in *A. nidulans. lox1* was expressed in all of the pCN4 transformants checked (e.g., TCN4.1, TCN4.4, TCN4.7 and TCN4.8). However, the expected size of *lox1* mRNA is approximately 2.8 kb and we found that the transformants only expressed a very small amount of a 2.8 kb transcript and instead that two smaller transcripts, presumably degraded *loxl* mRNA, also hybridized with the *lox1* probe.

Extracts of TCN4 strains did not show any reaction to the soybean Loxl antibody, nor did a ammonium sulfate precipitation of TCN4.8 proteins show any lipoxygenase activity as determined by standard lipoxygenase activities. Whether this is due to technique, degradation of the *lox1* mRNA in the fungus, etc., is unknown at this time.

Ascospore Development. All TCN3 and TCN4 transformants were impaired in their ability to produce ascospores. Eight strains (4 wildtype and 4 TCN3) were grown for 1, 2, 3, 4 or 5 days on ammonium tartrate MM and then switched to nitrate MM. After a total of 11 days of incubation, fungal plugs were cored from the plates and conidia and ascospores counted from each plug. Controls treatments were 11 days of growth on ammonium tartrate MM or 11 days growth on nitrate MM. Ascospore numbers of all TCN3 strains were ~10-100 fold less than TTA11 strains in all nitrate treatments. Growth in ammonium tartrate MM repressed ascospore formation in wildtype strains and there was no significant difference between ascospore numbers in TCN3 and TTAll strains in this treatment. A similar reduction of ascospore numbers were seen in TCN4 strains.

The lipoxygenase inhibitor gossypol did not return ascospore production to wildtype levels in either TCN3 or TCN4 transformants but did appear to stimulate ascopore production in general.

Mycotoxin analysis. Preliminary TLC results have shown that TCN4 transformants produce ST in MM (data not shown). Future experiments will determine if ST is affected when TCN4 and TCN3 strains are grown in *lox1* inducing medium.

DISCUSSION

Experiments carried out in our laboratory have shown that the soybean lipoxygenasel products, 13-(S)HPODE and 13-(S)HPOTE [Gardner 1995], decrease AF/ST production in *Aspergillus* spp. (Burow et al. 1996 and see companion poster by Burow et al.). Because similarly structured linoleic metabolites (collectively called Psi factors) are postulated to induce premature sexual sporulation in *A. nidulans* (Champe *et al.*, 1987; Mazur *et al.*, 1991), we postulated that the 13-(S)HPODE effects on mycotoxin production could be an indirect consequence of alternations in fungal development. To address this issue, we transformed *A. nidulans* (a sexually competent *Aspergillus spp.)* with the soybean *lox1* gene fused to inducible promoters, *(niiA(p)::lox1)* and *(alcA(p)::lox1).*

Induction of these promoters did result in *lox1* expression in *A. nidulans*. The fact that the transformants accumulated a smaller transcript than the expected size could indicate that a post-transcriptional processing of the transcript occurs. This putative post-transcriptional processing could explain why we did not detect lipoxygenase activity in these strains. At present, new *lox1*-expression vectors are being designed in order to improve the quality of the expression of this gene in *A. nidulans* and *A. parasiticus*.

In order to address our hypothesis that *A. nidulans lox1* transformants would be affected in sexual reproduction, we grew the transformants on solid medium under both inducing and non-inducing conditions and then compared ascospore production between *lox1* transformants and wildtype. The number of ascospores per surface unit was 10-100 fold less in all *lox1* transformants vs wildtype when grown in *loxl* inducing conditions. When grown in non-inducing conditions (ammonium and high glucose), wildtype and *lox1* transformants produced the same number of ascospores. When grown on MM plus peanut oil, a medium rich in the Loxl substrate linoleic acid, asexual sporulation was also reduced in the transformant TCN4.1 as compared to wildtype. We will determine if asexual sporulation is affected in the other *lox1* transformants when grown on lipid rich medium.

Gossypol--a toxic compound elaborated by cottonseed - has been used in other studies as a lipoxygenase inhibitor (Jensen *et at.* 1992). We added this compound to our medium in an attempt to recover the wildtype phenotype in the *lox1* transformants. Although the wildtype phenotype was not convincingly recovered in the *lox1* transformants, there was some sign of ascospore recovery in some pCn4 transformants. The one pCN4 transformant which showed no ascospore production, TCN4.8, also expressed the highest level of *lox1* transcript. Intriguingly, we found that 400 μM gossypol inhibited growth and stimulated cleistothecial and consequent ascospore production in the wildtype strains of *A. nidulans*. Garber and Cotty (1995) describe a similar effect on *A. flavus* where they found that comparable levels of gossypol inhibited growth but increased sclerotial and AF production in this asexual *Aspergillus* spp. The cleistothecium and sclerotium are the survival structures *of Aspergillus*

spp. and may represent analogous developmental responses to environmental cues in the Aspergilli.

In conclusion, the results of these experiments indicate a possible interaction or interference between soybean *lox1* products such as 13-(S)HPODE and fungal Psi factors with resultant impairment of fungal development (e.g., sexual and asexual reproduction). It seems probable that ST/AF biosynthesis and activation of sexual development could have some common pathway routes in a manner similar to the interaction between ST/AF biosynthesis and asexual development (Hicks *et al.*, submitted). One common motif in the experiments described in this poster is the presence of an active lipoxygenase and/or its linoleic acid products which are known to act as signaling molecules in other organisms. It is postulated that an endogenous *Aspergillus* lipoxygenase produces Psi factors (Champe *et al.*, 1987; Mazur *et al.*, 1991) and, indeed, lipoxygenase enzymes have been described in *Saccharomyces cerevisiae* (Shechter and Grossman 1983) and also recently in the genus *Fusarium* (Bisakowski et al., 1995). Also, the fact that the lipoxygenase inhibitor gossypol increases cleistothecial production, sclerotial and AF production suggests that a lipoxygenase-like enzyme may be necessary for these processes in the aspergilli. Regardless of whether the *Aspergillus* enzyme responsible for Psi factor formation is a lipoxygenase or a P-450 monooxygenase (as found in other organisms), we are interested in cloning this gene to determine if its function is important in *Aspergillus* development and subsequent mycotoxin biosynthesis.

FUTURE GOALS

1. To transform both *A. nidulans and A. parasiticus* with improved *lox1* expression vectors and to determine *lox1* effects on both development and AF/ST expression.

2. To analyze the lipid content of wildtype and *lox1* transformants (in collaboration with Dr. Harold Gardner, USDA-ASR, Peoria, Ill) in order to precisely identify which (if any) fatty acid-derived moieties are important in developmental/secondary metabolic signaling in *Aspergillus* spp.

3. Isolation and characterization of native *Aspergillus* lipoxygenases.

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EFFECT OF COTTON-LEAF VOLATILES ON MORPHOLOGY AND AFLATOXIN PRODUCTION OF *ASPERGILLUS PARASITICUS*

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The fungi *Aspergillus flavus* and *Aspergillus parasiticus* produce the hepatocarcinogenic, secondary metabolite, aflatoxin most commonly in cottonseed, corn, peanuts and treenuts. When aflatoxigenic strains of *A. flavus* are grown in the presence of specific cotton-leaf or maize volatiles, increases or decreases in aflatoxin production as well as variations in fungal growth are often observed (1,2). In some cases, growth is not significantly affected while aflatoxin biosynthesis is markedly inhibited (1). However, these studies have not revealed the mechanism by which these changes occur. Wilson et al (3) have shown that a maize volatile, β-ionone, negatively affected growth, aflatoxin production, sporulation and conidiophore development. This study seeks to understand the mode of action of cotton-leaf volatiles on morphological changes that are correlated with effects on fungal growth and aflatoxin production. For this purpose, four volatiles from almost 50 (1) were selected including two alcohols (3-methyl-l-butanol, and 1-nonanol, and two terpenes (limonene and camphene) volatile compounds. These metabolites affected either fungal growth or aflatoxin production.

The effects of volatile exposure at 0, 10, 25, 50, and 100 μl each of 3-methyl-l-butanol (butanol), nonanol, limonene, and camphene were evaluated. Butanol treated samples exhibited a decrease in radial growth that was directly proportional to butanol dosage. In addition, butanol treatment resulted in a loss of mycelial pigmentation that was accompanied by a marked decrease in sporulation. Samples grown in the presence of all doses of nonanol demonstrated uniquely aerial hyphae, and radial growth was inhibited 50 percent at 100 μl of nonanol; the conidia were tightly clustered on the condiophore as compared to control samples. Butanol enhanced aflatoxin production, nonanol had no significant effect. Limonene and camphene-treated samples yielded negligible differences in radial growth and morphology when compared to control; limonene drastically inhibited aflatoxin production, whereas camphene did not.

These results suggest that plant metabolites vary in their effects on fungal development and aflatoxin synthesis; some simultaneously affect both parameters whereas others affect either fungal development or aflatoxin synthesis.

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RELATIONSHIPS BETWEEN C_6-C_{10} ALKANAL AND ALKENAL VOLATILE CONTENTS AND RESISTANCE OF MAIZE GENOTYPES TO *ASPERGILLUS FLAVUS* AND AFLATOXIN PRODUCTION

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An association was found between C_6-C_{12} alkanal and alkenal volatile contents in several maize genotypes and aflatoxin contamination. Volatiles generated from untreated, ground, field resistant (R) maize kernels contained in an inverted lid of a Petri dish affected growth and aflatoxin production by *Aspergillus flavus* when spores were inoculated on potato dextrose agar in a closed, sealed Petri dish assay. Volatiles from the ground maize genotypes were purged onto Tenax columns and analyzed by GC/MS; C_6-C_{12} aldehydes were more prominent in the GC/MS total ion chromatograms of the (R) genotypes. Maize genotypes which exhibited a greater inhibition of aflatoxin production in the inverted lid assay also exhibited a larger concentration of linoleic acid in their fatty acid profiles. These results suggest the correlation of decay products of polyunsaturated fatty acids and plant disease resistance, and indicate that the lipoxygenase pathway may contribute to this resistance.

RELATIONSHIPS AND VOLATILE ASPECTS BETWEEN KERNEL POSITION ON THE MAIZE EAR AND AFLATOXIN FORMATION

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Volatiles produced by ground maize kernels from three different zonal areas of the maize ear resulted in varied influences on the growth of aflatoxigenic *Aspergillus flavus* and aflatoxin production in sealed, inverted lid, petri plate culture bioassay. Kernel volatiles from the medial zonal area of the maize ear resulted in 75% mean inhibition of *A. flavus* radial growth and a 76% mean inhibition of aflatoxin contamination. Kernel volatiles from the apical zonal area produced mean inhibitions of 19% radial growth and 33% aflatoxin contamination, and volatiles from the basal zonal area produced mean inhibitions of 23% radial growth and 37% aflatoxin contamination. Volatiles from ground kernels from apical, medial and basal zonal sections of the maize ear were purged on tenax columns and analyzed by GC/MS compounds highly toxic to *A. flavus* were concentrated in volatiles from ground kernels from the medial zonal area of the maize ear. These results demonstrate a relationship between volatile fungitoxic compounds generated from ground maize kernels extracted from separate zonal areas on the maize ear and aflatoxin contamination.

PHENOLIC SIGNAL MOLECULES INHIBIT AFLATOXIN BIOSYNTHESIS BY *ASPERGILLUS FLAVUS*

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Acetosyringone, syringaldehyde and sinapinic acid are phenolics which are shown to increase about ten times when metabolically active plants tissues are wounded. These phytochemicals have been demonstrated as signal molecules for the activation of virulene (vir) gene expression in *Agrobacterium tumefaciens*. When *A. flavus* invades wounded plant tissues, it is exposed to these signal phenolic molecules. In this study, the effects of these three phenolics, acetosyringone, syringaldehyde and sinapinic acid on the growth and aflatoxin biosynthesis of *A. flavus* were evaluated. Aflatoxins were analyzed by the technique of high pressure liquid chromatography (HPLC). The colored *NOR* mutant of *A. flavus* was also used in this study as a visual assay to examine the effect of phenolics on the early steps in aflatoxin biosynthesis.

All three phenolics inhibited aflatoxin biosynthesis of *A. flavus*. The order of inhibitory activity is acetosyringone > syringaldehyde > sinapinic acid. Acetosyringone was the most effective one, a concentration as low as 0.5 mM (94 ppm) resulted in about 23% reduction of aflatoxin. The concentration of acetosyringone for achieving 50% inhibition is 1 mM (182 ppm). As the concentration increased to 2 mM (364 ppm), 80% of aflatoxin biosynthesis was inhibited. In the case of syringaldehyde, significant inhibition of aflatoxin biosynthesis occurred at concentra- tion of 2 mM (364 ppm). Sinapinic acid was the least active phenolic, only 10% of aflatoxin biosynthesis was inhibited at concentration of 2 mM (448 ppm). The three phenolics did not have much effect on either its morphological growth or development of *A. flavus*.

The inhibitory effect of the phenolics on the NOR mutant of *A. flavus 827* can be quickly and easily visualized by the intensity of red orange color formed in the agar culture. As the concentrations of acetosyringone increased from 0.1 mM to 2 mM, the intensity of the red orange color decreased. The color was barely visible at 2 mM concentration. For the less effective phenolics, both sinapinic acid and syringaldehyde caused a reduction of red orange color in the agar culture when the concentration was 2 mM. The color changes can be unmistakably scored.

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